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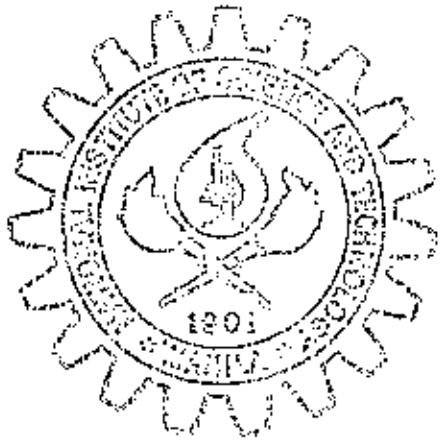

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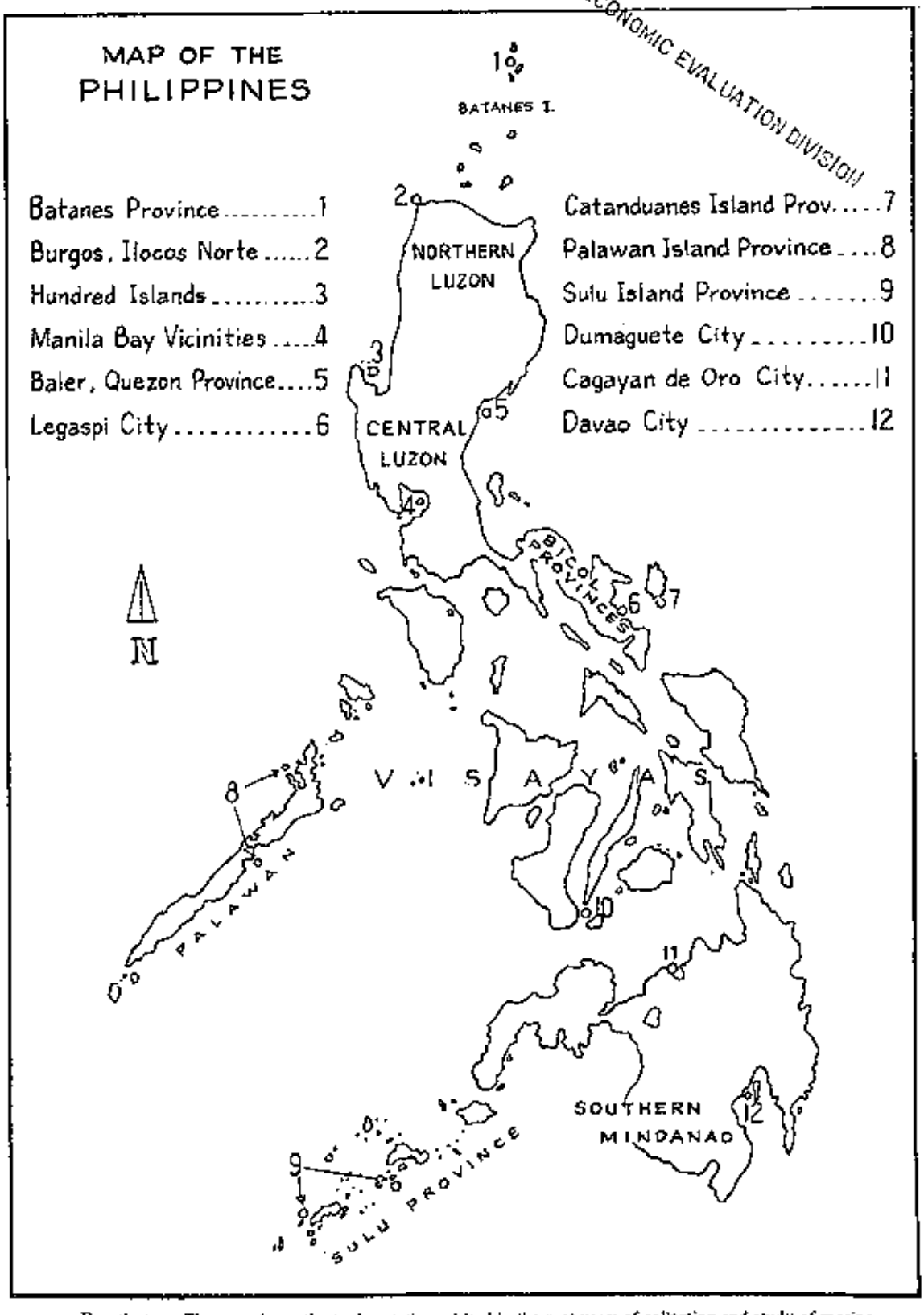

Frontispiece: The map shows the twelve stations visited in the course of collection and study of marine benthic algae.

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THE "MICROSCOPIC ALGAE IN THE HARD CORAL COMMUNITIES"[1]

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Quezon City

ABSTRACT

Four better known classes of the microscopic algae are represented in the study, namely: Myxophyceae, Rhodophyceae, Chlorophyceae, and Phaeophyceae, in that order of abundance and number of species (See Table 1). Representative samples of hard corals and edible fishes frequenting and or living in the coral communities are collected and with the algae were studied and represented in photographs. (Figures 1a and b).

The discussion centers on the significant role of the algae with which the other minute organisms in the corals were included and analyzed to explain the transfer of energy in the trophic level of food forms involved through the physio-biochemical processes. Such is a special activity in a complex recycling. A brief presentation of some selected background work with relevance to the research is reviewed.

Goggles easy to use in diving and improvised bows and arrows suited together to catch the fishes in the corals are suggested for distribution freely. It should introduce a practical and least expensive rural technology in far-away places generally much neglected.

INTRODUCTION

After more than three decades with the experience of the author to study the benthic marine algae of the Philippines from 1946 to the present, Frontispiece to shows the 12 stations studied, it was thought to be advisable

[1]The report is based on a similar research at Puerto Galera, Oriental Mindoro, site of the Marine Biological Station of the University of the Philippines. The two subsequent progress reports, one at Tacloban City and Vicinities, 1978 and the second at Iloilo City and Vicinities, 1979 furnishing more materials of study to complete the investigation. The financial support was given by the Office of the Research Coordination, University of the Philippines at Quezon City.

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to make an additional study of finally the microscopic algae associated with the hard coral communities. It is indeed noteworthy to consider the food relationship of these algae to the fishes, and other organisms in the corals. There are several coral communities observed growing in the deeper littoral zones towards the fringes of many continental shelves where plenty of fishes are usually observed to be present. The other coral communities generally have very much less fishes or sometimes none at all.

One of the major causes of fish abundance in the hard coral communities which should attract them is undoubtedly the presence of food. At large including man the vast majority of the organisms in nature have been virtually attracted to food. Although usually out of many observations only around three or four coral communities out of several communities in an area would show the presence of fish abundance. Another possible cause of similar attraction is the behavioral response of some fishes towards the corals which is a problem left to be studied and solved by the psychologist, ichthyologist, and more likely the ecologist interested in the behavioral sciences.

OBJECTIVES

1. To study the taxonomy of the microscopic algae, the hard corals and the fishes attracted to these corals in the Philippines.

2. Later, and as early as possible, to study the other associated microscopic organisms which should include the delicate phytoplankton and zooplankton before their extinction from the effects of pollution.

3. Extend similar studies in other parts of the country to include the observations before the submission of the final terminal report.

4. And finally, to recommend the results of the investigation as an additional approach at large to the increase of marine fish production suited to the eco-social conditions of the poor natives living along the remote seacoasts.

FIELD TRIPS

During the past three years, three separate regions have been visited, namely: The University of the Philippines Marine Biological Station at Puerto Galera, 1977; Tacloban City, the site of the Junior College of the University of the Philippines, 1978; and Iloilo City, another site of a Junior College of the University, 1979. Figures 2, 3 and 4. As much as possible collection of the hard corals and fishes observed to be staying in the coral

communities were made in the deeper littoral zones. The Department of Zoology, College of Arts and Sciences of the University and the Bureau of Fisheries and Aquatic Resources were requested for their cooperation to identify the corals and fishes, respectively.

The observations and results of the research at Puerto Galera, 1977 (Figure 1a and b) were utilized as the main reference of the two subsequent reports, one at Tacloban City and Vicinities, 1978 and the other at Iloilo City and Vicinities, 1979. (See Figures 3 and 4, respectively).

For almost a quarter of the present century from 1946, at least 12 major field trips were made in collecting and studying the benthic marine algae. Each collection lasted from 5 days or more during the low tide periods in each of the 12 stations. It was observed that the different locations represent the general distribution of the hard corals which form communities. The west waters of the South China Sea and the East Pacific Ocean are traversed by many seas crossing the country to form virtually a continuous eco-system where many of the corals and the edible fishes are interrelated and form similar species in a common environment, Frontispiece.

The Definite Localities of Field Trips. Since the investigation is a new approach to research in the Philippines, it was necessary that the first locality to be studied is at Puerto Galera, Oriental Mindoro where the writer had sufficient experience during at least fifteen years of summer classes in the site of the Marine Biological Station of the University. The observation of hard coral communities and the fishes swimming in and around the corals were usually a special attraction to the students during the field trips. The first study therefore of the microscopic algae in the hard coral communities and the edible fishes attracted by them was made and used as the basic reference of the two subsequent patterns of investigation. Separate samples of hard corals in the communities and fishes in these communities are shown in photographs, Figures 1a and b. The table on page 6 is an enumeration of the microscopic algae observed in the coral communities, their general relative amount of growth is shown as x x x abundant, x x average and x rare.

INVESTIGATION AND DISCUSSION

Field trips were done seaward of the deeper zones where the fishes are generally observed to frequent the hard coral communities. Studies in the field and laboratory undoubtedly revealed that several fishes seen and caught in the corals besides being attracted by many other factors must primarily be induced by the available food. If this is true, from what major sources should the food come from?

The vegetarian fishes such as the siganids and the parrot fishes are known to depend for their food from submerged plants including the soft algae by nibbling or grasping the surface of the plant body before swallowing them in fragmentary forms. The microscopic algae growing in the corals are easily nibled piece by piece although in several instances there are forms swallowed wholly with the unicellular organisms before reaching the digestive tract. On the other hand, the diatoms together with the various planktonic organisms passed through the gill slits leading to pouches and depository pockets of some fishes before they join as parts of the other food fragments swallowed by the fish. The non-vegetarian fishes also in the corals obtained their food from the complex processes which will be discussed later.

Past investigations local and abroad revealed only very limited references in the fish productivity of the hard coral communities. And yet statistics would reveal that 10% of the world consumption of fish thrive in the corals. The location of the Philippines in the tropics and its unusual-unique geographical distribution composed of seven thousand and one hundred islands have formed naturally several seas and other smaller bodies of marine water areas where the fishes are abundant throughout the year. The temperature generally does not differ very much during the year within the average limit of 5°C in the surrounding waters.

On the other hand, very few papers if any of the investigations on corals have included the microscopic algae in these corals as parts of the study. The majority of the other studies on the marine benthic algae would include only the more conspicuous species for taxonomy. Many other algae inhabiting the corals are too small to attract or to be seen by the taxonomist during the collections.

L. Egerod, 1971 and 1974, in her algal studies reported in Phycologia and Botanica Marina were included some microscopic algae living in the hard corals without any mention of their relationship. The two publications were limited to taxonomy. D. Squiles in Nature, 1964, emphasized predators biting and rasping the tips and polyps of the corals which include the associated microscopic algae. The report made mention only of some correlations between the species of fish and the types of corals. S. Brawlex, et al., 1977 limited their work by indicating only the heavy grazing of fishes towards the peripheral portions of many coral communities. One would incline to guess that such grazing could be attracted by the microscopic algae.

Assimilation efficiency of its food in a vegetarian fish, *Siganus spinus* was studied by Bryan in 1975. Muscatine on the other hand studied in 1969 the photosynthetic products of zooxanthellae in the coral polyps. The same author observed the symbiotic relationship of the algae and some lower invertebrates. There was none available where the algae were revealed to be playing the primordial role in the formation of food compounds as major attraction to the fishes. Finally, Alcala in his work on the aspects of the coral reefs in Sumilon Island observed several edible fishes but there was no mention of the microscopic algae living in the corals.

The algae as it is true with the higher plants manufacturing their food in photosynthesis consequently accumulate the energy of the sun in various food compounds which become available in many ways to other organisms. The abundance of the blue-green algae has shown undoubtedly to be primordially significant in the food chain complex of the different organisms thriving in the coral communities. They are usually the first setlers of a barren habitat by virtue of being very simple and ubiquitous organisms. The vegetarian fishes feeding directly from the microscopic algae function normally in their metabolism which they need for growth while the other fishes which are not herbivours might in one way or the other survived in the coral communities intimately in the presence of other organisms. Therefore, the presence of the blue-green algae caused the other organisms including the microscopic animals to live together in the coral communities. It is bio-nutritive activity of the complex recycling processess taking place in the relationships of trophic levels among the organisms in the coral environment. This interdependence of the various organisms during their metabolic activities shall need more detailed research. It gives an opportunity to the zoologist and biochemist to participate in the new investigation in order to bring about a more equitable solution in the present investigation.

Recent informations have been known of the artificial corals dropped in the shallower parts of the continental shelf in Taiwan which was observed later to be the favorite habitat of several edible fishes. Similarly in Malaysia, the old tires of motor vehicles dropped in the seaward area of the deeper littoral zones attracted also the fishes after two or three years. The fishes are observed also and equally abundant in the immediate vicinities of the sunken tires. With closely related observations, R. Tsuda revealed in 1974 a study of the algal growth succession on the artificial reefs in a marine lagoon at Guam. Evidently, similar succession of growth in the ecology of microscopic algae in Taiwan and Malaysia was observed where the blue-green algae undoubtedly must have played a significant role. The edible fishes in both Taiwan and Malaysia supplemented at least 10% of the fish food of the poor people in the rural areas along the seacoasts.

There are many other possible localities where similar investigations can be done. As shown in the Frontispiece not less than twelve easily accessible sites in the Philippines had been observed by the author which show similar coral populations. The country due to its geographical formation is typically composed of definitely very poor rural areas. It is these places where the people along the shores must depend upon the edible fishes caught in the corals.

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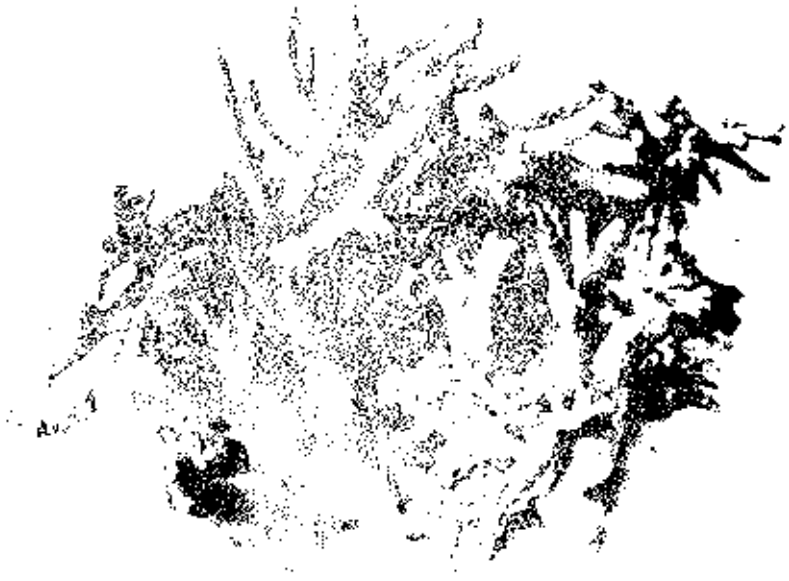
13. _____. 1980. The Microscopic Algae in the Hard Coral Communities: Two progress reports to the Office of the Research Coordination of the University of the Philippines for faculty development, namely-living Microscopic Algae in the Hard Coral Communities of Tacloban City and Vicinities, 1978 and the same title repeated at Iloilo City and Vicinities, 1979.

Table 1. *The microscopic algae living in the corals carefully detached and studied to represent the prepoderance of the algae in the majority of hard coral communities.*

ALGAE	Myxophyta	Chlorophyta	Phaeophyta	Rhodophyta
Lyngbya martensiana	XX			
Spirulina major	X			
L. confervoides	XXX			
Cladophora		XX		
Polysiphonia				XX
Herposiphonia				XXX
Zoochorella		XX		
Schizothrix	X			
Ceramium				X
Gleoetrichia	XX			
Oscillatoria bonnemaisonii	X			
Oscillatoria spp.	XX			
Lyngbya spp.	XX			
Bryopsis		X		
Gelidiella acerosa				X
Sphacelaria mucifera			X	
Ectocarpus			X	



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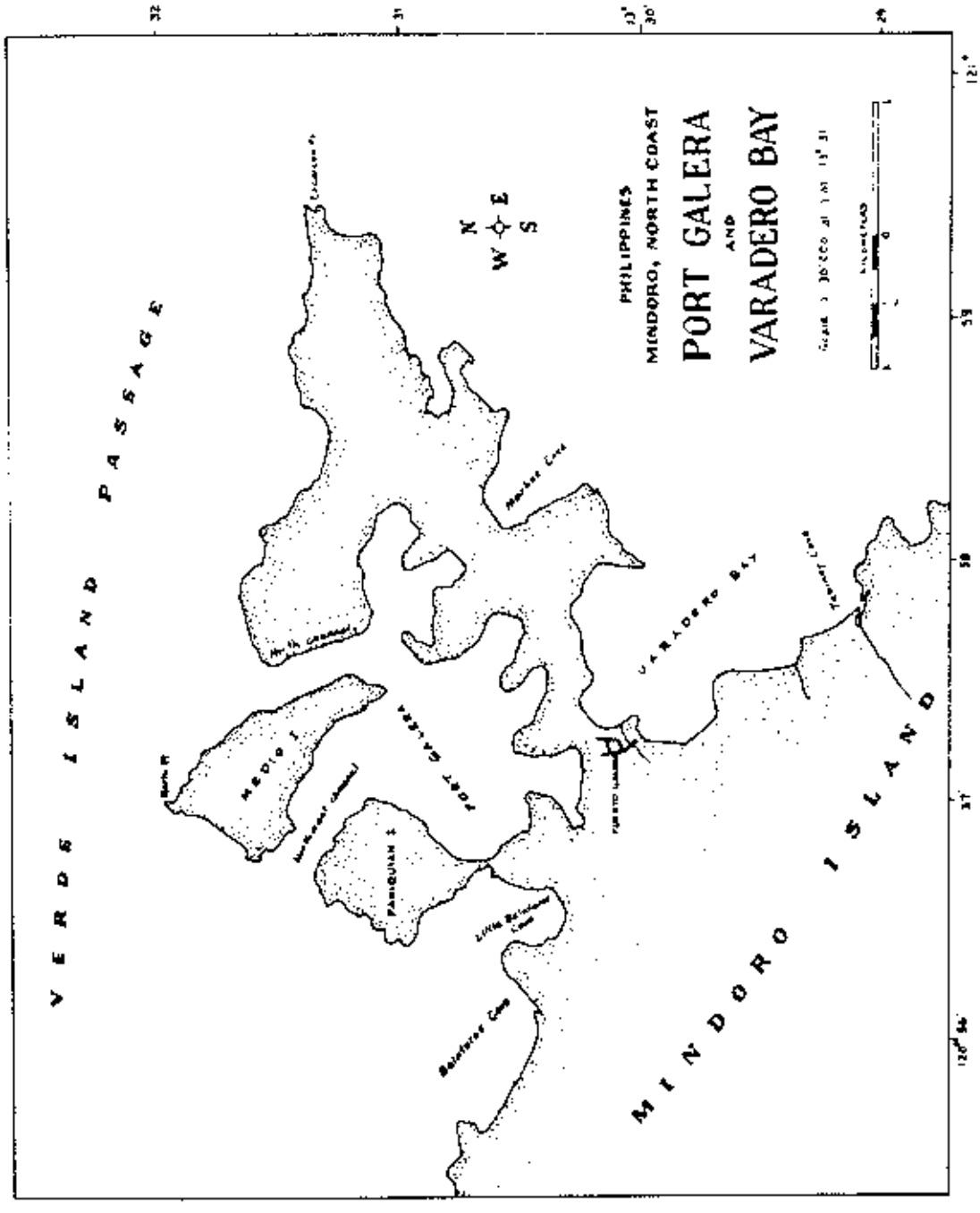
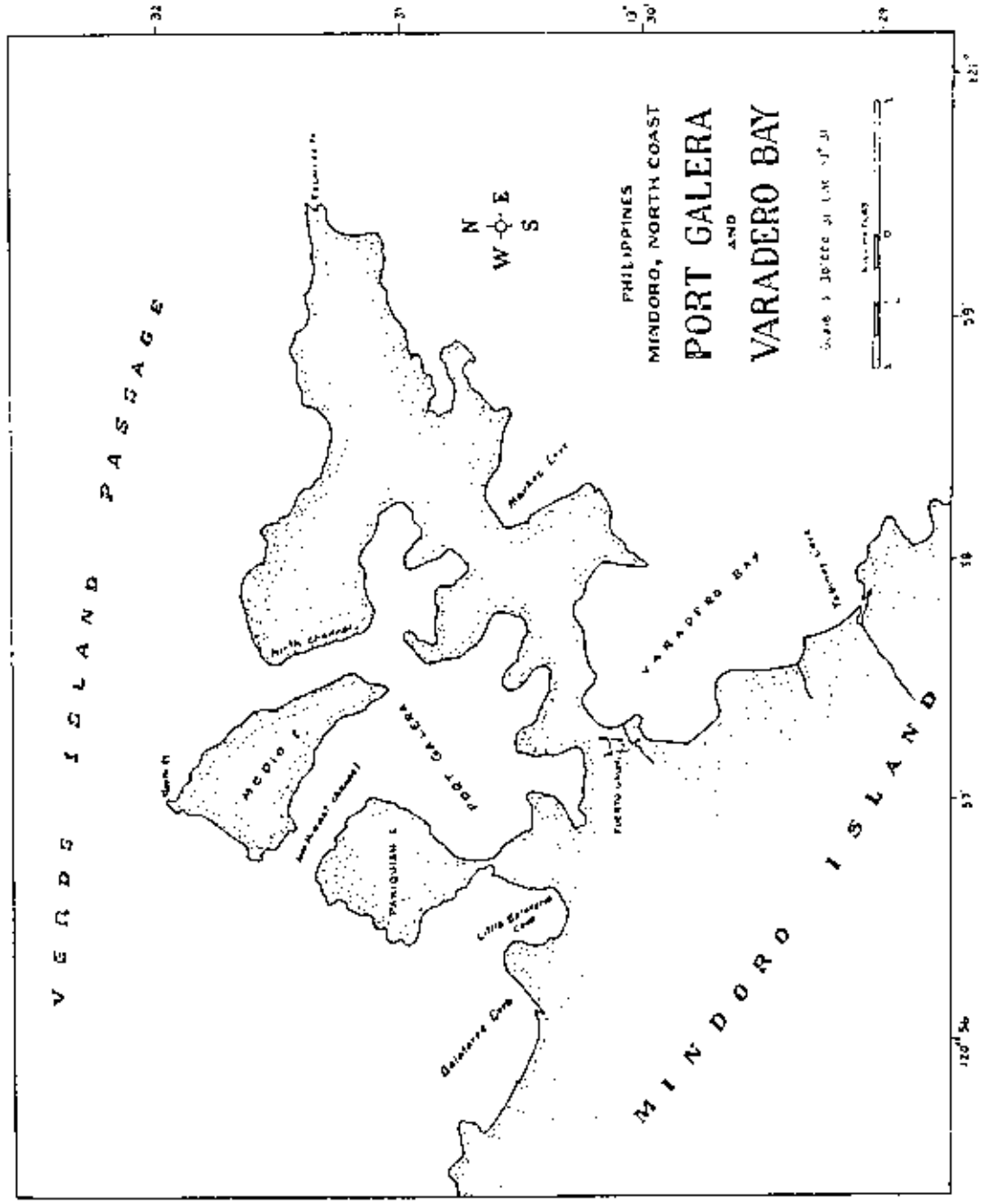


Figure 2. Map of Puerto Galera, Oriental Mindoro where the original study of the microscopic algae and the hard coral communities was made, 1977. The Study was the reference investigation of the other regions.



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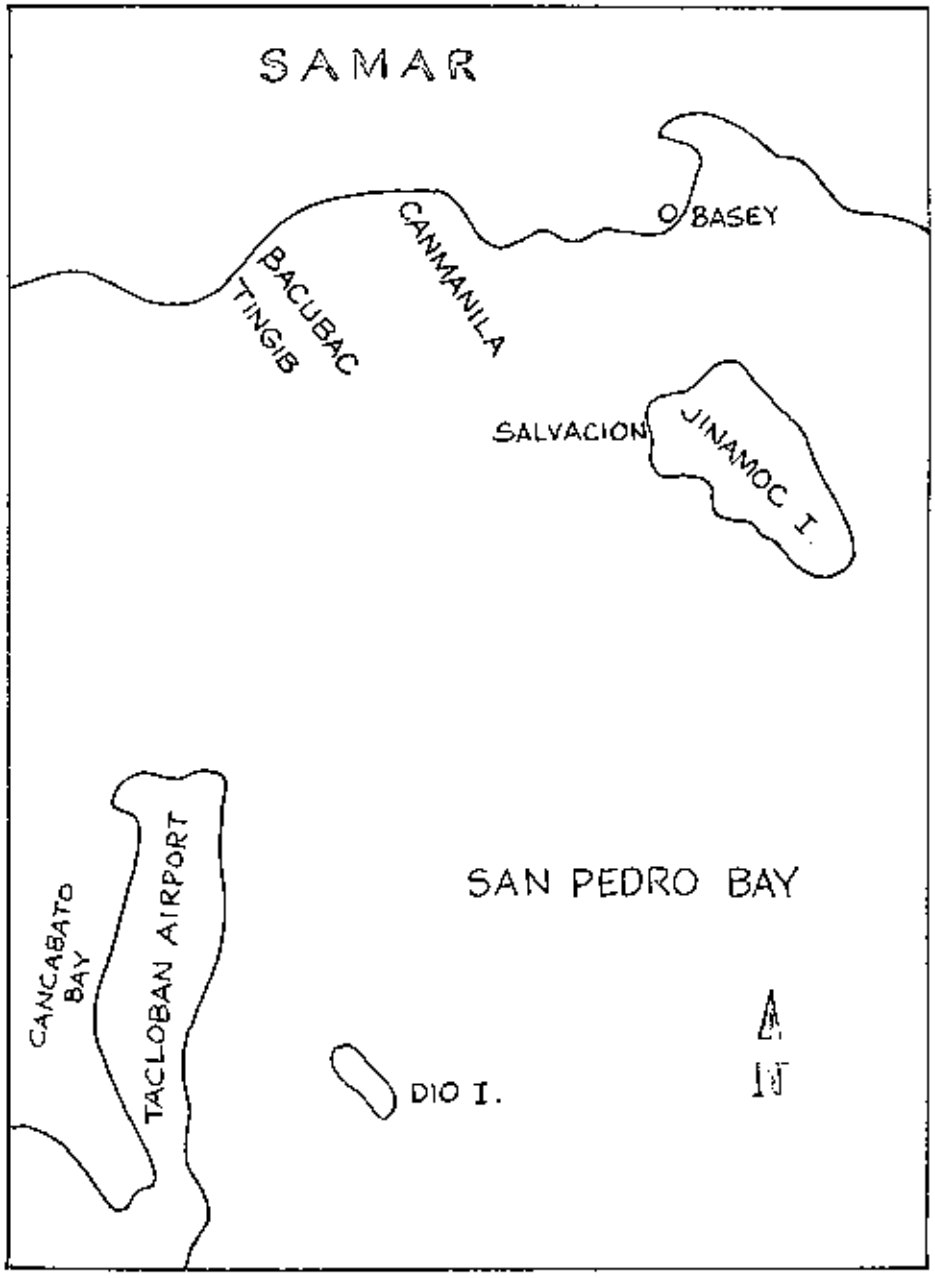

Figure 3. Map to show Tacloban City, in part. First in a series where the microscopic algae and the hard coral communities was studied, 1978.

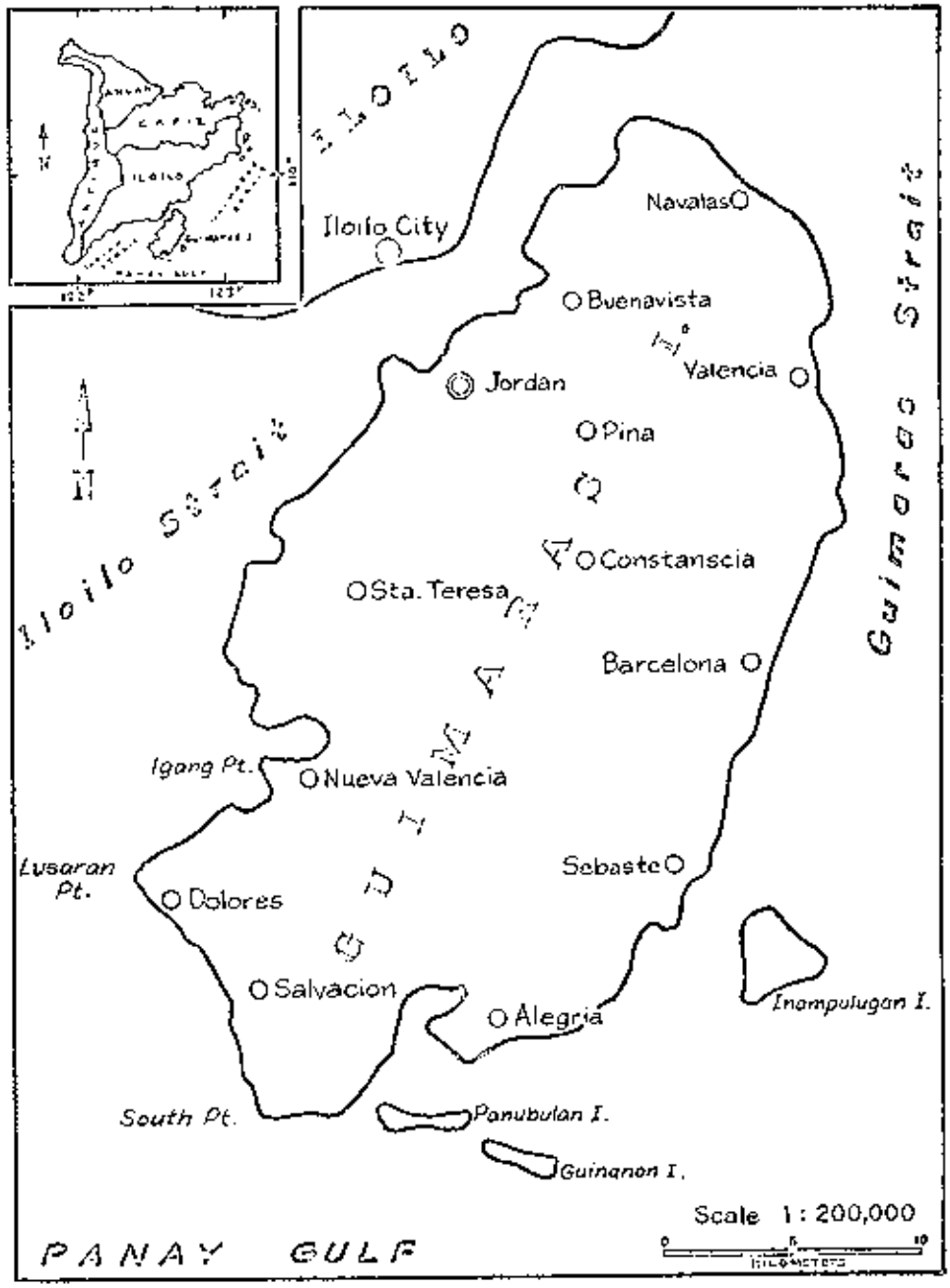

Figure 4. Map to show Iloilo City, in part. Second in a series where the microscopic algae and the hard coral communities was studied, 1979.

SKIM MILK-COAGULATING ACTIVITIES OF ENZYMES PRODUCED BY PHYCOMYCETOUS FUNGI*

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ABSTRACT

An exploration of local Phycomycetous fungi flora was conducted, to discover species or strains capable of synthesizing potent milk-clotting enzyme that can be utilized as a substitute for calf's rennet in cheese making from cows milk. Of the sixty seven isolates of saprophytic Phycomycetous fungi belonging to genera *Rhizopus, Mucor* and *Cuninghamella* some strains had already been found to produce milk-clotting enzymes. These strains are capable of clotting skim milk in suitable concentrations of calcium ions in 4 to 10 minutes.

INTRODUCTION

The milk-coagulating preparation known among the cheese makers as rennet consists chiefly of rennin, the enzyme that causes curdling of milk. It is normally obtained from the fourth stomach of a suckling calf of a cow. In the recent past the supply of rennet for cheese making could not cope with the rising demand for cheese by the rapidly increasing numbers of cheese consumers. To solve the shortage of rennet by obviating the slaughtering of costly calves, many scientific investigators explored the living universe of plants, animals, fungi and bacteria for potential source of enzymes capable of curdling milk.

The exploration of microbial world for sources of milk-clotting enzymes was intensified during the past eight years primarily by Japanese investigators[1-5] and secondarily by American and European researchers[6-11]

*This investigation, designated NRCP Project No. LG-5 was supported by grants-in-aid of the National Research Council of the Philippines and was undertaken in the BRC Laboratory with the gracious permission of the Commissioner, National Institute of Science and Technology, NSDB, Manila.

**To whom correspondence is to be directed.

who showed that microorganisms producing enzymes with milk-clotting properties varied from minute bacteria to macroscopic mushrooms.

The present investigation on microbial source of milk-coagulating enzymes was inspired by the work of Arima and Iwasaki[1] who showed that a Phycomycete mold *Mucor pussillus* Lindt. possessed a potent milk clotting enzyme for cheese making experiments.

This work aims to screen different species of fungi, both local and foreign species, for a possible milk-clotting activity. The microbial exploration will center chiefly on saprophytic molds belonging to class *Phycomycetes*, a group of fungi with typically unsegmented, vegetative structure.

MATERIALS AND METHODS

Collection of mold-bearing materials. – Mold-bearing samples consisting of soil from rhizosphere of cereal grass, rotting fruits, decomposing garbage, trash and manures, compost, deteriorating plant parts, spoiled foods and vegetables, were collected from different markets in Metro Manila, Rizal, Laguna and Batangas provinces. These are used as materials from which the rennet-producing strains were isolated. The molds were induced to grow vigorously, sporulate by placing portions of the mold bearing samples in sterilized culture plates lined inside with sterile moist filter paper. With the distinct features of the forms of Phycomycetes it was not difficult to recognize the molds belonging to this group by the characteristic growth and appearance of their vegetative and fruiting structures as revealed under the microscope.

Isolation of the molds in pure culture. – Isolation of the mold was done by streaking loopfuls of the spore suspension in sterile water on suitable solidified potato-dextrose agar medium in sterile plates. Another method is by pour-plate dilution of the spore suspension in culture plates containing similar medium. Transfers were made after an incubation of two days from uncontaminated, vigorously growing colony into test tube agar slants.

Cultivation of the molds in suitable solid organic substrate. – Among the different solid organic substrates that had been tried in cultivating the molds, copra meal was found to be fairly satisfactory. Its purpose is to stimulate them to produce larger concentration of the potent milk-clotting enzyme. As substrate it could be completely permeated by the mold mycelia and transformed into a coherent cake-like material in 3 days.

Preparation of the enzyme extract. – The fungi impregnated copra meal substrate was sliced into smaller pieces. A volume of 175 ml sterile tap

water was poured into flask computed to make a 10% enzyme extract. The molded mass was sliced under submerged conditions with the aid of an improved long stemmed spatula. The mixture was transferred into an electrically operated Waring Blendor wherein it was comminuted and homogenized to attain maximum extraction of the water soluble enzyme. After straining it through thick cheese cloth the filtrate was further clarified by centrifugation with the International Centrifuge at more than 2,000 rpm for 15 min to separate the solid particles.

Precipitation of the crude enzyme by salting out with ammonium sulfate. – The crude enzyme was easily precipitated by salting-out with ammonium sulfate. Some water-miscible organic solvents like methanol, ethanol and acetone had been tried as precipitants. None of them could produce an amount of precipitate as large as that obtained by ammonium sulfate treatment. Precipitation was enhanced by subjecting ammonium sulfate treated extracts to low temperature (5-10°C). Six grams of ammonium sulfate was added to 10 ml of the enzyme extract. The precipitate containing the enzyme was separated by centrifugation at 4,000 rpm for 25 minutes. It is then dissolved immediately in 10 ml water. One ml of the enzyme solution in this case, represents 1/2% of the enzyme extract obtained from a 3-day old culture of the mold in a 20 g sample of the copra meal substrate.

Method of assaying for milk-clotting activity. – The milk-clotting activity of the crude enzyme was determined by using the modified Berridge[2] method. It is done by mixing, 1 ml of the enzyme solution produced by different mold strain with 10 ml of the test substrate consisting of 10% cow's skim milk powder fortified with $CaCl_2.2H_2O$ of known molarity, and pasteurized at 65°C for 1 hour. The mixtures were kept at room temperature and at 45-50°C. The time of the formation of the curd fragments was measured with the aid of a stop watch. One unit of enzyme activity was taken to be that which clotted 10 ml of milk in 10 minutes.

Four trials were made in the determination of milk-clotting activities of the skim milk solution with different calcium (from $CaCl_2.2H_2O$) concentration namely , 0.01 M, 0.05 M and 0.001 M at various temperatures. This was done to know the effect of calcium++ on milk-clotting activity of the crude extract.

RESULTS AND DISCUSSION

Sixty seven Phycomycetous fungi isolates were screened for milk-clotting activity. Those that produced inappreciable amount of precipitate when salted out with ammonium sulfate were discarded. Seventeen were found to produce substantial amounts of the enzyme precipitate after

treatment with $(NH_4)_2SO_4$. These were the isolates used in succeeding tests for milk-clotting activities (Table 1). It will be noticed that out of 17 mold isolates tested, B-415, M-14, M-15, R-107 and R-106-2 appear to be promising. A camera-lucida drawings of the 3 of them were shown in figures 1, 2 and 3. The 5 promising isolates mentioned above were cultivated at room temperature (27-32°C) in copra meal substrate wherein complete permeation of the mold mycelia could be seen after 72 hours of incubation (Fig. 5). The enzyme solutions prepared from these promising isolates, except for the isolate R-107, cause curdling of milk in only 4 to 10 min at room temperature and 3 to 13 min at 45-50°C.

As can be seen from table 1, 16 isolates produced clotting activity in all Ca^{++} concentrations tried at room temperature, but at a higher temperature several isolates did not clot the milk with 0.05M and 0.001 M $CaCl_2.2H_2O$. Isolate R-108 gave a poor clotting activity when it showed a much delayed clotting time (244 min) in tubes containing 0.01 M Ca^{++} concentration at 45-50°C and its failure to clot milk with the different Ca^{++} concentration tried at room temperature.

Influence of Ca^{++} concentrations namely; 0.01M, 0.05M and 0.001M by referring to table 1, shows that milk-clotting activity of the mold enzymes increased with increasing concentrations of the Ca^{++}. Shorter clotting in skim milk solutions with high molarity were produced than those with lower molarity of $CaCl_2.2H_2O$. The results shown in table 1 served to verify the findings of Iwasaki and Arima[9] and Kawai and Mukai[5] that the presence of Ca^{++} in a skim milk solutions accelerate the milk clotting activities of the microbial rennet.

At room temperature (27-32°C) the formation of curd was observed in almost all skim milk solutions to be slow. Very few (only the 5 promising strains) curdled at a shorter time. At 45-50°C, curdling was noticed in tubes containing 0.01M $CaCl_2.2H_2O$ to be very fast (except for R-108). Close examinations of the data in table 1 shows that milk-clotting were, in many cases, inhibited at 45-50°C where the concentrations of Ca^{++} were lower than 0.01M $CaCl_2.2H_2O$.

While shortness of clotting time could be an index of greater milk-clotting activity, this alone would not make a good criterion for active milk clotting strains. The milk-clotting activity is also measured by the firmness and the consistency of the curd, by the degree of peptonization, i.e. digestion of the curd by the proteolytic ferments into a soluble and diffusible substance, chiefly, peptone,; by the amount of the whey produced or the balance between milk-clotting activity and proteolytic activity. The milk-clotting activities of all the mold strains tested (table 1) had been observed in close association with proteolytic activity. This phenomenon is not new.

There was a previous attempt to prove that this 2 associated activities observed in rennet were 2 distinct enzymes or 2 functions of only 1 enzyme. No general agreement was reached on this question. Nevertheless, Arima and Iwasaki[4] observed that nearly all proteolytic enzymes are able to curdle milk.

In cheese manufacturing the use of fungi strains that produce milk-clotting enzymes with higher proteolytic power is objectionable. It is believed that they always yield cheese products with soft texture and objectionable flavor and taste. The desirable fungi strains are those that produce a large concentration of the enzymes with potent milk-coagulating property and weak proteolytic activity. The 5 mold isolates, B-415, M-14, M-15, R-106-2 and R-107 which were found in screening tests to be promising are to be studied further. One or 2 with superior, stable features will be selected for use in cheese manufacture from animal milk.

CONCLUSION

The study shows that the exploration of our local microflora is worthwhile continuing. It is not only a means of unearthing fungi capable of producing enzymes with excellent milk-clotting properties for use in cheese manufacture, but also a way of discovering superior fungi strains that can be harnessed to produce substances of great economic or industrial value.

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Table 1. *Milk-Clotting Activities of Enzymes Produced by Phycomycetous Fungi*

ISOLATE NUMBER	GENERA	*MILK-CLOTTING ACTIVITY (Average of 4 trials)*		
		In 10% skim milk solutions with increasing molarity of $CaCl_2.2H_2O$	At room temperature (27-32°C)	At 45-50°C
		0.	Minutes	Minutes
B-415	Rhizopus	0.001 M	0*	0*
		0.05 M	4	10
		0.01 M	4	10
B-416	Mucor	0.001 M	72	0
		0.05 M	54	0
		0.01 M	40	7
B-417	Mucor	0.001 M	81	0
		0.05 M	79	22
		0.01 M	39	2
B-418	Mucor	0.001M	48	0
		0.05 M	45	12
		0.01 M	9	9
B-419	Mucor	0.001 M	162	0
		0.05 M	120	0
		.01 M	45	20
B-420	Mucor	0.001 M	50	55
		0.05 M	45	40
		0.01 M	40	16
L-301-1	Rhizopus	0.001 M	219	0
		0.05 M	171	0
		0.01 M	114	69
L-301-2	Mucor	0.001 M	219	0
		0.05 M	171	0
		0.01 M	114	23
M-2	*Mucor subtilis simus*	0.001 M	96	45
		0.05 M	69	20
		0.01 M	36	16
M-14	Rhizopus	0.001 M	10	4
		0.05 M	8	4
		0.01 M	4	4
M-15	*Rhizopus Nivcus*	0.001 M	7	5
		0.05 M	6	5
		0.01 M	4	3

Table 1: *Milk Clotting Activities of Enzymes Produced by Phycomycetous Fungi*

ISOLATED NUMBER	GENERA	*MILK CLOTTING ACTIVITY (Average of 4 trials)*		
		In 10% skim milk solutions with increasing molarity of $CaCl_2.2H_2O$	At room temperature (27-32°C)	At 45-50°C
M-16	*Rhizopus stolonifer*	0.001 M	56	0
		0.05 M	55	0
		0.01 M	20	5
R-104	Cunning-hamella	0.001 M	14	15
		0.05 M	14	15
		0.01 M	14	15
R-106-1	Mucor	0.001 M	122	0
		0.05 M	94	58
		0.01 M	42	31
R-106-2	Cunning-hamella	0.001 M	5	10
		0.05 M	4	10
		0.01 M	4	10
R-107	Rhizopus	0.001 M	88	13
		0.05 M	66	12
		0.01 M	46	7
R-108	Rhizopus	0.001 M	0	0
		0.05 M	0	0
		0.01 M	0	244

* By 0 minute = no clotting was observed

ILLUSTRATIONS

The photomicrographs and photographs by Mr. Ricardo Marquez, Technical photographer, Division of Documentation, NIST-NSDB.

PLATE 1

Figure 1. Camera–lucida drawings of a Phycomycetous fungi. Isolate no. R-106-2 (Cunninghamella) approximately X175. Note the fruiting structure with central or primary conidiophores which have developed terminal spore heads upon which form globose conidia. The lateral branches of the conidiophores occur singly and form whorl terminating into vesicles covered with conidia.

Figure2. Photograph of a camera-lucida drawings of an alga-like fungi. Isolate no. R-107 (Rhizopus) approximately X175 from a 48-hour old plate culture on potato-dextrose agar. This is another fungi that can cause clotting of cow's milk in 4 to 10 minutes at 45 to 50°C. The picture shows:

a) rhizoids
b) sporangiophore
c) sporangium
d) ruptured sporangium wall
e) collumella
f) collarette
g) sporangiospores

PLATE 2

Figure 3. Photographs of a camera-lucida drawing of another Phycomycetous fungi. Isolate no. B-415 (Rhizopus) showing the development of the fruiting structure (sporangiophores) and sporangia and the rhizoids.

Figure 4. Milk-clotting activity of the five promising mold isolates. The enzyme prepared from them clotted the skim milk solution fortified with M/100 $CaCl_2.2H_2O$ in 4 to 10 min at 45 to 50°C.

Figure 5. Five isolates cultivated in copra meal substrate for 72 hr, B-415, M-14, M-15, R-106-2 and R-107. (Note the mycelial growth of the mold in copra meal substrate)

Fig. 1

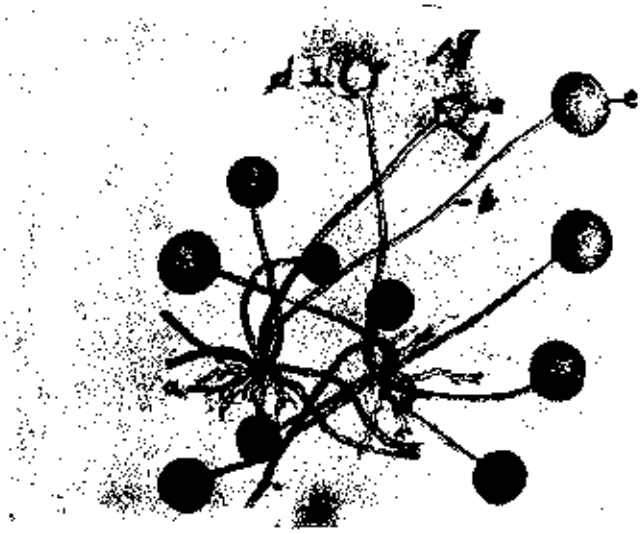

Fig. 2

PLATE 1

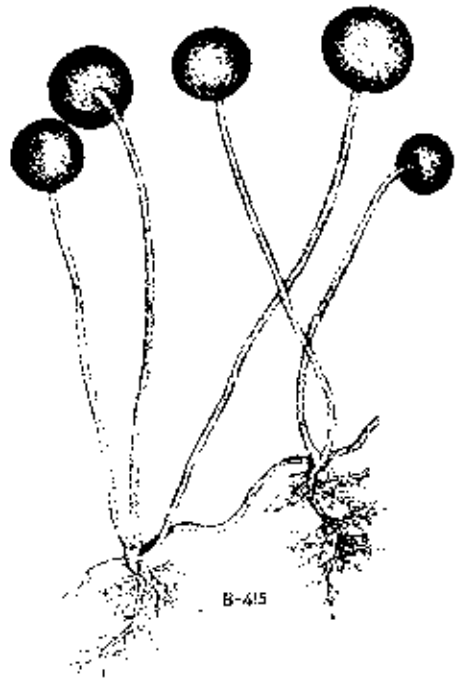

Fig. 3

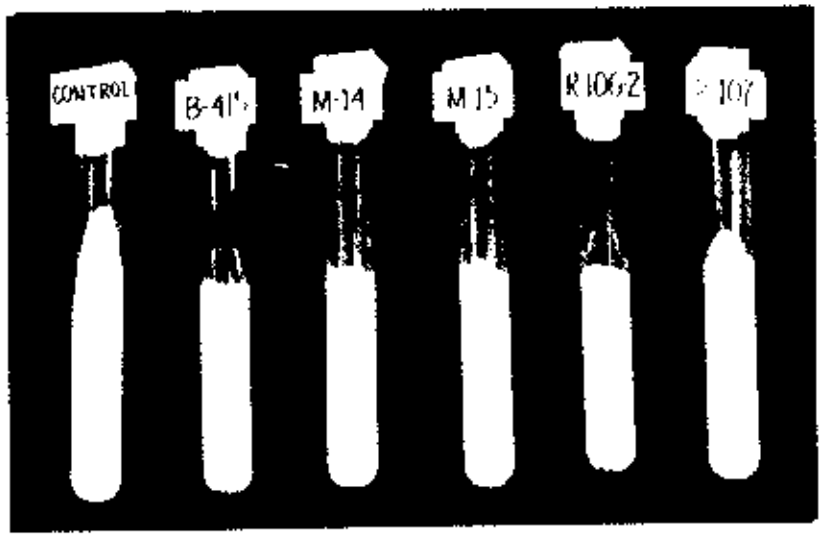

Fig. 4

PLATE 2

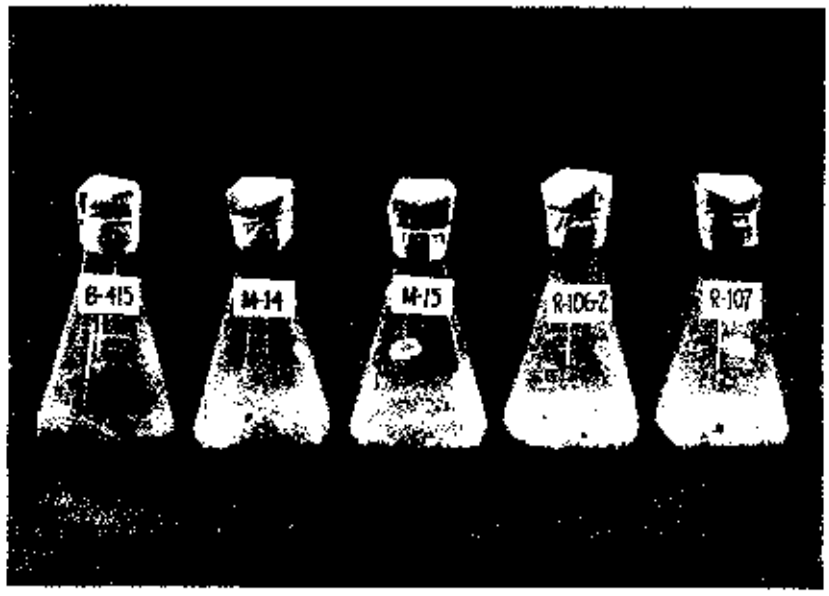

Fig. 5

PRODUCTION AND UTILIZATION OF TANNIN FROM IPIL-IPIL [*Leucaena leucocephala (Lam) de Wit*] BARKS*

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ABSTRACT

Tannins from ipil-ipil *[Leucaena leucocephala (Lam) de Wit]* barks were extracted by a 6 stage countercurrent method at 80°C with 1:8 bark to water ratio. The yield of extract obtained was 70.98%, based on the oven-dry weight of the raw material. The head liquors and washings obtained were combined and spray-dried in the Bowen laboratory spray-dryer. Tannin-formaldehyde adhesives were prepared from the extract and used to bond 3-ply white lauan veneers. The panels did not delaminate in the 4-hour cyclic boil test for Type 1 plywood of the US Commercial Standard. The addition of 10-40% phenol-formaldehyde resin or 5-30% resorcinol-phenol-formaldehyde resin improved the water resistance of the adhesive. When fortified with 40% phenol-formaldehyde resin, the tannin adhesive met the requirements of the test for Type I Plywood of the US Commercial Standard. With resorcinol-phenol-formaldehyde resin, 25% was found necessary for the tannin adhesive to pass the test for type 1 Plywood of the US Commercial Standard.

INTRODUCTION

A. *Scope of the Study*

This study deals with the determination of the yield and quality of tannin obtained by hot-water extraction of ipil-ipil [*Leucaena leucocephala (lam) de Wit*] barks taken from Catanauan, Quezon and the development of tannin-formaldehyde adhesive from the spray-dried extract. The effect of the different levels of paraformaldehyde, pH, pressing time, pressure, and the effect of the addition of fortifying resins on the glue shear strength of the tannin adhesive were investigated.

B. *Importance of the Study*

Ipil-ipil trees are widely distributed in the Philippines and is presently being used for fuel and for reforestation. *Ipil-ipil* barks contain more than

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10% tannin and because of the abundance of this tree species, it may be profitable to extract from the barks tannins, as a source of phenolic adhesive and for tanning of leather.

Because of the rising cost of phenol due to the continuing increase in the price of oil, *ipil-ipil* tannins may be used as an alternative source of phenol to lessen the cost of phenol-based adhesive. In the leather industry, *ipil-ipil* tannin if found suitable for tanning leather may be used as a substitute for the imported mimosa tannin.

C. *Review of Literature*

Tannins are extracted from barks by either the batch or open-vat system, using the countercurrent method. Several important variables that affect the yield and quality of tannin extracts are temperature, time of leaching, liquid to solids ratio, nature of solvent, particle size and number of batches or vats in the system. Kurth and Smooth [1, 2] investigated the effect of these variables.

Many researchers in the field of tannins have succeeded in formulating adhesive from different tannin extracts. As early as 1950, Dalton[3] developed tannin-formaldehyde adhesives from 6 species including commercial wattle (*Acacia molisima*) extract and extracts from the bark of wood of *Callitris calcarata, C. glauca, Eucalyptus redunca, E. Grebra* and *E. considentiana*. The adhesives produced with extracts from *A. Mollisima, C. calcarata, C. glauca, and E. Grebra* set at moderate temperature forming with wood strong water-resistant bonds. Adhesives prepared with the tannins from *A. mollisima and C. calcarata* set at room temperature to give bonds with strength somewhat lower than those obtained from typical commercial adhesives but having water resistance superior to the cold-set urea-formaldehyde adhesives. Brandt[4] developed plywood adhesive from mangrove tannin extracts. Thermo-setting tannin adhesives from quebracho and wattle were also formulated by Knowles and Gardner[6] in their study on adhesive from bark extracts.

Plomley[7] showed that the incorporation of small proportions of various synthetic resins with tannin formaldehyde resin improved its bond strength. He fortified mangrove and wattle tannin with phenolic and resorcinol resin to produce plywood which gave high shear values and wood failure, and withstood immersion in boiling water for 72 hr giving conformity to the latest weather and boil-proof requirement. In 1958, Narayanamurti[8] discovered that tannins from Bruguiera species are excellent materials for wood adhesive. In the same year, Herrick and Bock[9] combined polymethylol phenols with bark extracts to produce exterior plywood type adhesive.

The effect of soluble salts in decreasing the gelation time of wattle and mangrove tannin solutions with formaldehyde was reported in 1959[10]. Improved bond quality was obtained when 5% of manganese acetate was added to a wattle tannin adhesive.

Nillis and Urbach[11] used catechin as a model compound to study the tannin-formaldehyde reaction. The study of the reaction of mangrove and wattle-polyphenols with formaldehyde at various pH and under different conditions of temperature and time were also undertaken[12]. They concluded that formaldehyde uptake at pH 8 and 100°C in 3 hrs gave a better indication of the efficiency of polyphenol as an adhesive than did the determination of Stiasny polyphenols.

Herrick and Conca[13] developed a cold-setting adhesive of high strength by combining the spray-dried sodium salt of an ammonia extract of Western hemlock bark with resorcinol-formaldehyde resin and additional formaldehyde.

Tannin resins derived from black wattle tannins have been used commercially in Australia, since 1963[14]. It is understood that production has increased substantially since then. In 1966[15] a report was made on studies of physical and chemical properties of wattle-tannin adhesives.

Chestnut wood tannin extract was used commercially in Malaysia in 1973 when there were a shortage of phenol because of the oil crisis[16]. Norsechem, a joint venture between Malaysian and Norwegian groups, which produces synthetic urea-formaldehyde, phenol-formaldehyde and other adhesive resins for the wood working industry, introduced a phenol-formaldehyde resin modified with chestnut wood tannin extract for the plywood industry. The chestnut tannin extract chemically replaced part of the phenol up to 50 percent with satisfactory results in phenolic glue manufacture.

D. *Objectives of the Study*

The objectives of this study are to prepare quality tannin extracts from *ipil-ipil* barks and develop a tannin-formaldehyde adhesive from the extracts for plywood manufacture.

E. *Time and Place of Study*

This study was undertaken at FORPRIDECOM, NSDB, College Laguna from December 1975 to January 1977.

MATERIALS AND METHODS

Materials:

1. Ipil-ipil barks obtained from Catanauan, Quezon were air-dried, chipped and ground in the Wiley mill to pass a 10-mesh screen.
2. Test panels

   31 cm x 15.2 cm x 15.2 cm white lauan veneers for faces and cores.

The panels or veneers were selected according to the straightness of their grain. The moisture content of the veneers was about 9 percent.

Methods:

Analysis of bark: The bark was analyzed for tannin content, using the ALCA method and the method of Stiasny.

Laboratory-Scale Extraction. The temperature employed was a continuous six-stage countercurrent process as described in technical Note No. 120[17]. The temperature of extraction was 80°C with bark to water ration of 1:8. A definite amount of ipil-ipil bark (600 g) was placed in each of 6-4 liter beakers and water was placed in the first beaker. The beaker was placed in a water bath maintained at 60 °C, for one hour with constant stirring. After one hour, the tannin liquor was drawn/off the first beaker and fresh water was added to the partially spent material and the extraction repeated. At the end of the hour, the extract was drawn off the first beaker and fresh water was added to the partially spent material and the extraction repeated. At the end of the hour the extract was drawn off from the first beaker for extraction of the fresh material in the second beaker. Fresh water was again added to the first beaker and the extraction repeated until there were 6 units in operation.

The six-stage countercurrent method was repeated until three batches of head liquors were collected. The first batch of spent materials with six solvent-treatments was removed from the system.

Analysis of the Spent Barks and Head Liquors. The spent materials were air-dried and ground in the Wiley mill to pass a 10-mesh screen. These were analyzed for total solids, soluble and insoluble solids, tannins and non-tannins in accordance with the procedure of the American Leather Chemists Association. The Stiasny number was determined. The head liquors were analyzed using the same procedure.

Stiasny Method of Tannin Determination. In a 250 ml. Erlenmeyer flask fitted with a reflux condenser, 50 ml. of tannin solution, 5 ml. of

concentrated hydrochloric acid and 10 ml. of formaldehyde solution (37% by weight) were mixed. The mixture was boiled for 30 minutes and filtered while hot through a weighed glass filter crucible with a pore size of 20-30 microns. The precipitate was washed with 200 ml of hot water and dried to constant weight at 100-105°C.

Spray-drying of the Extract. The combined head liquors and washings were spray-dried in the Bowen laboratory spray dryer with inlet temperature of 149°C and outlet temperature of 93°C. The rate of drying was 3.18 l/hr.

Preparation of the Glue Mixture

A. *With varying proportion of paraformaldehyde*

Twenty grams spray-dried *ipil-ipil extract (M.C. 6.55%)* were mixed with 20 ml water and 1 gm coconut shell flour. Paraformaldehyde (varied at 5-20% of the weight of tannin) was added to mixture and the mixture applied on white lauan veneers.

B. *At different pH*

Twenty grams spray dried *ipil-ipil* tannin were mixed with 20 ml of water. Varying amounts of 20% NaOH soln. were added to adjust the pH of the extract to 6, 7, 8, and 9. Coconut shell flour (1 gm) and paraformaldehyde (10% of the dry tannin) were added to each mixture at different pH. The glue was applied on white *lauan* veneers.

C. *At different pressures*

Twenty grams of tannin extract were mixed with 20 ml water. Coconut shell flour (1 g) and paraformaldehyde (1 g) were added to the tannin extract. The adhesive was applied on white *lauan* veneers and the assembly were pressed at different pressures. (8.79, 10.55 and 12.30 kg/sq cm) for 10 minutes.

D. *With phenol-formaldehyde resin as fortifier*

Twenty grams tannin extract were mixed with 20 ml water. To this, were added varying proportions of phenol-formaldehyde resin (5, 10, 15, 20, 25, 30, 35, and 40% of the weight of tannin). Coconut shell flour (1 g) and paraformaldehyde (1 g) were added to the tannin extract. The adhesive was applied on white *lauan* veneers.

E. *With different pressing time*

Twenty grams tannin extract were mixed with 20 ml water and 1% phenol-formaldehyde resin weighing 10% of the tannin content was added.

Coconut shell flour (1 g) and paraformaldehyde (1 g) were added to the mixture. The adhesive was applied on white *lauan* veneers. Each assembly was pressed at a different period of time (2,4,6,8, & 10 min).

F. *With resorcinol-phenol formaldehyde resin as fortifier*

Twenty grams tannin extract were mixed with 20 ml water and varying proportions of resorcinol-phenol formaldehyde resin (5, 10, 15, 20, 25, and 30% of the tannin content) were added to the extract. Coconut shell flour (1 g) and paraformaldehyde (1 g) were added to the mixture. The adhesive was applied on white *lauan* veneers.

Preparation of Phenol-Formaldehyde Resin

Phenol-formaldehyde resin was prepared in the laboratory following the procedure of Plomley[18]. Phenol was reacted with formaldehyde in the molar ratio of 1:1.5 with sodium hydroxide as catalyst. The initial concentration of NaOH was 2% of the weight of phenol but this was increased during the course of the reaction to 5% in order to keep the resin in solution.

The materials were reacted together at 85°C under a reflux condenser with constant stirring. The reaction time was 5½ hr. After reation, the resin was colled to room temperature and sodium hydroxide was added to bring the NaOH content to 6.2% based on the resin solids

Preparation of Resorcinol-Phenol-Formaldehyde Resin

The method of Plomley[18] was used in the preparation of resorcinol-phenol formaldehyde resin. One mole phenol was mixed with one mole of formaldehyde (37% solution) and NaOH catalysts was added. The mixture was further heated at 90°C for 40 min after which it was cooled to ambient temperature as rapidly as possible.

Preparation of test panels

The adhesive mixture was applied on both sides of the core veneer with a brush. The amount of spread was 20 g of the adhesive mixture per 0.093 sq m single glue line. The face and core veneers were assembled with their grains at right angles to each other. The assembly was allowed to stand for 1 hr before the application of pressure. Each panel was pressed for 10 min at 160°C under a pressure of 12.30 kg/sq cm (175 psi).

The panels were conditioned for 1 week before testing for glue line shear strength by the standard procedure.

Evaluation of Bond Strength

Shear test – The shear test specimens were cut from the test panels according to the procedure of the U.S. Commercial Standard for Hardwood Plywood (CS35-11)[19]. The shear tests were conducted using the Reichle plywood shear testing machine, the rate of loading ranging from 272.7-295.4 kg/min.

Water resistance test – To test whether the glue line was weakened by water, the test for water resistance for Type II plywood of the Japanese Export Standard[20] was used. Four test speciments taken out of each test piece were submerged in hot water at a temperature of 63°C (± 3°C) for a period of 3 hr. The specimens were then soaked in water. While the specimens were wet the shear test was conducted.

Cyclic boil test – For the tannin-formaldehyde adhesive fortified with phenol-formaldehyde adhesive and resorcinol-phenol formaldehyde adhesive, the cyclic boil test of the American Commercial Standard for hardwood plywood was applied. The shear specimens were boiled in water for 4 hr and then dried for 20 hr at a temperature 63°C (± 3°C). These were boiled again for 4 hr, cooled in water, and subjected while wet to the shear test.

RESULTS AND DISCUSSION

Results

Table 1 shows the tannin analysis of *ipil-ipil* barks.

Table 2 shows the tannin analysis of the spent *ipil-ipil* barks.

Table 3 shows the yield of extract obtained from the 6 stage counter current extraction at 80°C.

Table 4 presents the tannin analysis of the head liquor.

Table 5 gives the tannin analysis of the extract before and after spray-drying.

Table 6 shows the effect of the levels of paraformaldehyde on the bond strength of the adhesive.

Table 7 gives the effect of pH on the bond strength of the adhesive.

Table 8 is a summary of the effect of pressure on the bond strength of *ipil-ipil* tannin-formaldehyde adhesive.

Table 9 shows the effect of the addition of phenol-formaldehyde resin on shear strength of *ipil-ipil* tannin-formaldehyde adhesive.

Table 10 shows the effect of pressing time on the bond strength of *ipil-ipil* tannin-formaldehyde adhesive.

Table 11 is a summary of the effect of pressing time on the bond *strength of* ipil-ipil tannin formaldehyde adhesive fortified with 10% phenol-formaldehyde resin.

Discussion

Ipil-ipil bark contains more Stiasny tannins than tannins reactive with the hide powder. The tannin extract may be more promising as an adhesive rather than for the tanning of leather because the Stiasny number is 84.57 while the tannins reactive with the hide powder is only 64.28%. The yield of extract obtained by extracting the tannins using a 6-stage countercurrent method at 80°C ranged from 68.48-73.71% based on the raw bark. The yield of Stiasny tannins is more than the yield of tannins reactive with the hide powder. The head liquor obtained had a concentration of 4.78%. There was very little insoluble solid material and the tan to non-tan ratio of the extract was 1.97:1. A good quality tannin extract should have a tan to non-tan ratio of more than 1 and preferably 2, 0[19].

Spray-drying adversely affected the quality of the extract because the tan to non-tan ratio decreased to 1.32:1 and the Stiany number decreased from 84.57 to 81.85. The decrease in tannin content may be caused by the high temperature during the spray-drying process. The Stiasny number of the extract may be comparable to the Stiasny number of black wattle (acacia mollissima) tannin which is 90-93. The tannin-formaldehyde adhesive formulated from the extract of black wattle is fully water-proof and resists the 72-hr boil test of the Australian Standard for Plywood[20].

In the formulation of *ipil-ipil* tannin formaldehyde adhesive, 10% parafonnaldehyde based on the dry tannin weight was found necessary to produce a good bond.

The natural pH of the extract was at pH 5. At this pH the adhesive produced was rough in texture. The addition of NaOH to adjust the pH to 6 produced an adhesive with a smooth consistency and which can easily be spread with a brush. The viscosity of the adhesive increased as more NaOH was added to adjust the pH to 7, 8 and 9. With increase in pH the usable life of the adhesive decreased. At pH 6, the pot life was 4½ hr, at pH 7, 1 hr 40

min, at pH 8, 1 hr and at pH 9 only 40 min. The plywood panels delaminated during the wet shear test when the pH of the adhesive was 9.

As the pressure was raised from 8.79 kgs/sq cm (125psi) to 12.30 kgs/sq cm (175 psi), there was an increase in glue shear strength.

Without the addition of any fortifying resin, *ipil-ipil* tannin-formaldehyde adhesive resisted the cyclic boil test so it is superior to the commercial urea-formaldehyde resin. The addition of as much as 10% phenol-formaldehyde resin improved the water resistance of the adhesive but as much as 40% was found necessary to make the plywood panels pass the specification for type I plywood. With resorcinol-phenol formaldehyde resin the minimum amount necessary to improve bond quality was 5%, but as much as 25% was needed to make the panels meet the requirement of the US Commercial Standard for type I plywood.

Ipil-ipil tannin-formaldehyde adhesive fortified with 10% phenol-formaldehyde adhesive requires a short curing time. At 160°C, a 2-min curing time seemed adequate.

CONCLUSION

The yield of tannins obtained by extracting *ipil-ipil* barks using a 6-stage countercurrent method at 80°C was 71% based on the oven-dry weight of the bark. The extract had a high Stiasny number (84.57) and a tan to non-tan ratio of 1.97:1. The tannin formaldehyde adhesive prepared from the spray-dried tannin extract exhibited glue shear strength superior to the commercial urea-formaldehyde resin. With addition of 40% phenol-formaldehyde resin or 25% resorcinol-phenol formaldehyde resin the water resistance of the adhesive improved. It also contributed to its conformity to the specification for type I plywood (fully waterproof) of the US Commercial Standard.

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Table 1. *Tannin analysis of Ipil-ipil (Leucaena leucocephala) Bark*[a]

Total solids,[b] %	23.54
Soluble solids,[b] %	22.88
Insoluble solids,[b] %	0.66
Tannin,[b] %	11.26
Non-tannin,[b] %	11.62
Stiasny No.[b] %	20.81
Purity	49.21

[a]Analysis by ALCA method.
[b]Figures are based on moisture-free bark.

Table 2. *Tannin Analysis of Spent Ipil-ipil (Leucaena Leucocephala) barks*[a]

Total solids,[b] %	6.66
Soluble solids,[b] %	6.50
Insoluble solids,[b] %	0.16
Tannins,[b] %	4.34
Non-tannin[b] %	2.16
Stiasny[b]	4.98

[a]Analysis by ALCA method
[b]Figures are based on moisture-free bark.

Table 3. *Percent Yield base on Results Obtained in Tables 1 & 2*[a]

Total solids,[b] %	70.98
Soluble solids,[b] %	71.59
Insoluble solids,[b] %	72.22
Tannin,[b] %	59.41
Non-tannin,[b] %	81.15
Stiasny number[b]	76.05

[a]Analysis by ALCA method.
[b]Figures are based on moisture-free bark.

Table 4. *Tannin Analysis of Head Liquors*[a]

	Head Liquor 1	Head Liquor 2	Head Liquor 3	Average
Total solids,[c] %	4.51	5.28	4.56	4.78
Soluble solids,[b] %	98.49	93.69	98.49	96.87
Insoluble solids,[b] %	1.51	6.31	1.51	3.11
Non-tannin,[b] %	33.69	33.26	30.92	32.62
Tannin,[b] %	64.80	60.43	67.57	64.28
Stiasny number	85.44	82.08	86.25	84.57
Purity	65.79	64.49	68.61	66.29

[a]Analysis of ALCA method.
[b]Figures are based on total solids.
[c]Based on head liquor.

Table 5. *Tannin Analysis of Extracts Before and After Spraydrying*[a]

	Before[b] Spray drying	After[c] Spray drying
Total solids,[c] %	4.78	100
Soluble solids,[c] %	96.89	99.39
Insoluble solids,[c] %	3.11	0.61
Non-tannin[c] %	32.62	42.85
Tannin[c] %	64.78	56.54
Stiasny number	84.57	81.85

[a]Analysis by ALCA method.
[b]Averages results from tannin analysis of head liquors, table 3.
[c]Figures are based on total solids.

Table 6. *Effect of the Levels of Paraformaldehyde*[a] *on Bond Strength*

% Paraformaldehyde	Dry shear - WF (kg/sq cm)	Wet shear – WF (kg/sq cm)
5	20.74-15	17.43- 0
10	22.64-20	18.42-15
15	18.91-20	14.24-15
20	24.11-20	19.75-10

[a]Percentage weight of dry tannin.
(Gluing conditions: White *lauan* veneer, 3 ply 2.5 mm
glue spread – 490 g/m^2, pressing time, 10 min
Close assembly time – 1 hr, pressure = 12.30 kg/sq.cm
The second = figure under "Shear" refers to percent wood failure.

Table 7. *Effect of pH on the Bond Strength of the Adhesive*[a]

PH	Dry shear - WF (kg/sq cm)	Wet shear - WF (kg/sq cm)
5	17.36-10	10.68-0
6	20.38-20	12.79-0
7	14.91-0	9.98-0
8	16.17-0	11.95-0
9	5.97-0	0

[a]Similar gluing conditions as in Table 5.

Table 8. *Effect of Pressing Pressure on Bond Strength of Tannin Formaldehyde Adhesive from Ipil-Ipil*[a]

Pressure kg/sq cm	Dry shear - WF kg/sq cm	Wet shear - WF kg/sq cm
8.79	10.55-0	8.58-0
10.55	13.28-10	9.21-0
12.30	14.62-10	10.55-0

[a]Similar gluing conditions as in Table 5.

Table 9. *Effect of Phenol-Formaldehyde Resin on Shear Strength*[a]

% P-F resin	Dry shear-WF (kg/sq cm)	Wet shear[b]-WF (Kg/sq cm)	Wet shear[c]-WF (kg/sq cm)
0	19.40-5	16.52-0	13.14-0
5	19.48-55	15.61-0	9.49-0
10	15.45-50	10.96-55	10.96-17
15	16.73-50	15.45-64	13.35-40
20	22.84-60	16.87-50	10.75-25
25	15.04-35	11.45-80	15.04-40
30	22.84-60	18.84-25	11.38-13
35	21.30-35	18.13-63	13.21-20
40	19.54-100	16.34-90	13.71-75

[a]Similar gluing conditions as in Table 5.
[b]Soaked for 3 hr in hot water at 63°C then immersed in cold water and while wet the shear test was conducted.
[c]Cyclic boil test – The specimens were boiled in water for 4 hr and then dried for 20 hr at a temperature of 63°C (± 3°C) and then boiled in water for 4 hr, cooled in water, and then subjected while wet to the shear test.

Table 10. *Effect of Pressing Time on Ipil-ipil Tannin-Formaldehyde Adhesive*[a]

Time of Pressing	Dry shear - WF kg/sq cm	Wet shear WF (Cyclic Boil Test)
2 mins.	16.24-30	11.45- 0
4 mins.	17.93-30	14.41- 0
6 mins.	15.33- 8	11.32-25
8 mins.	15.11-30	11.46-10
10 mins.	18.06-30	17.29- 4

[a]Similar gluing conditions as in Table 5 except for the time of pressing.

Table 11. *Effect of Resorcinol-phenol-formaldehyde Resin on the Bond Strength of Ipil-ipil Tannin-formaldehyde Adhesive*

%	Dry shear-WF (kg/sq cm)	Wet shear[b] WF (kg/sq cm)
0	15.11-20	8.44- 0
5	20.04-25	13.71-10
10	14.94- 0	12.65- 0
15	14.48- 5	13.29- 0
20	14.41- 5	12.30- 0
25	20.04-90	15.04-65
30	16.73-75	14.91- 5

[a]Similar gluing conditions as in Table 5.

[b]Cyclic boil test – The specimens were boiled in water for 4 hr and then dried for 20 hr at a temperature of 63°C (± 3°C) and then boiled in water for 4 hr cooled in water and then subjected while wet to the shear test.

EGG SHELL FORMATION IN CARMYERIUS SYNETHES (FISCHOEDER, 1901) AND GASTROTHYLAX CRUMENIFER (CREPLIN, 1847) (DIGENEA: GASTROTHYLACIDAE)*

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Early works on egg shell formation in trematodes include those of Ujiie (1936) in *Clonorchis sinensis* (Cobbold, 1875) Looss, 1907; Dawes[1] in *Hexacotyle extensicauda* Dawes,[1] Kouri and Nauss[2] and Stephenson[3] in *Fasciola hepatica* L. Based on histochemical observations, they concurred that the bulk of the egg shell was formed by globules contained in the vitalline cells. Yosufazai[4] believed that the egg shell is secreted by the Mehlis' gland as shown by the argentophility of the egg shell of *Fasciola hepatica* and the detection of a hyaline secretion from the Mehlis' gland which was similarly argentophilic. Clegg[5] obtained lipoprotein by chemical analysis of fractions prepared from extract of Mehlis' gland of *Fasciola hepatica*. Smyth[6] however doubted the exact role of the Mehlis' gland in egg shell formation. He said that, in some species, two types of gland cells in Mehlis' gland produce mucus and lipoprotein. A uterine involvement of egg shell formation has been reported by Coil and Kuntz[7] in the trematode *Syncoelium spathulatum* Coil and Kuntz[7]. Smyth and Clegg[8] in their reviews of egg shell formation of trematodes and cestodes, confirmed the views of Ujiie (1936), Dawes[1], Kouri and Nauss[2] and Stephenson[3] that the shell globules from the vitelline cells in *Fasciola hepatica* are concerned in the formation of the egg shell. They remarked that "the egg shell formation in parasitic platyhelminths presents complex problems, many of which little work has been carried out".

Smyth[9] found malachite green and methyl green pyronin of special value in following the fate of the globules during egg shell formation. Johri and Smyth[10] recommended this method as a routine formation in trematodes. Madhavi[11] successfully employed permanganate aldehyde

*Part of a thesis submitted to the College of Arts & Sciences (Graduate Program), University of the Philippines in partial fulfillment of the requirements for the degree of Master of Science under the direction of Dr. Carmen C. Velasquez, Professor and Chairman, Dept. of Zoology, College of Arts and Sciences, University of the Philippines. Inquiries are to be directed to Dr. C. C. Velasquez.

fuchsin technique in following the fate of the vitelline globules in egg shell formation in the amphistomes *Diplodiscus mehrai* Pande, 1937; *Paramphistomum cervi; Cotylophoron cotylophorum* and *Gastodiscus secundus* Loose, 1907 and found the vitelline globules and egg shell positive for the test. He also pointed out that this method can be used with confidence to stain keratin in amphistome egg shell. He used sodium sulfide solution solubility test to confirm the presence of keratin. Histochemical tests conducted on the egg shell of the sanguinicolid trematode, *Orchispirium heteroviteliatum* by Madhavi and Hanumantha Rao[12] showed an elastic type of protein and not keratin or sclerotin as previously described for other trematodes. Burton[13] observed in his study on the fine structure of the Mehlis' gland of the frog lung fluke, *Haematoloechus medioplexus* that membranous bodies from this gland appear to become fused with the vitelline globules forming egg shell in the lumen of the ootype and proximal uterus. The most recent addition to our knowledge on this subject is that of Irwin and Threadgold[14] who obtained evidence by electron microscopic sudies that egg shell in *Fasciola hepatica* is formed from shell globules of the vitelline cells thus conforming previous findings. To enhance our knowledge of the biology of amphistomes, egg shell forma-necessity be studied.

Since antigenic properties are also linked with the egg shell in trematodes, knowledge of the egg shell formation is of significance.

MATERIALS AND METHODS

Specimens were fixed either in formal-saline, 10% formalin or Bouin's solution. After fixation, they were processed by the paraffin method. The following techniques were employed: 1-malachite green technique[9], 2-methyl green pyronin technique[9], 3-permanganate aldehyde fuchsin reaction[11], 4-bromphenol blue method[15] modified by Johri and Smyth[10], 5-Morel-Sisley diazotization method (Lillie, 1957) in Pearse (1961) and 6-ferric chloride test,[16,8].

The Goddard and Michaelis technique (1934) (Madhavi, 1971) was used for the solubility test to determine the keratin nature of the egg shell. Gomori's formula (1950) (Pearse, 1961) for the aldehyde fuchsin stain was adopted. Rapid staining with freshly prepared solution was done since this alters its property with age. Rosa's (1953) (Pearse, 1961) aldehyde fuchsin stain in the dry form was also employed to overcome the instability of the stain.

Where counterstaining was desired, 1% orange G in absolute alcohol was used. In all cases, sections were cut 5-8 microns thick. Three trials were conducted for each of the test.

RESULTS AND OBSERVATIONS

The vitelline follicles and the egg shell in both *C. synethes* and *G. crumenifer* were positive for the malachite green technique. (Plate I, figs. 1 & 2, Plate II, figs. 7 & 8). The Mehlis' gland utering wall and other parts were negative.

With permanganate aldehyde fuchsin stain, the vitelline globules and egg shell for both species were strongly positive for the test. (Plate I, figs. 3 & 4; Plate II, figs. 9 & 10).

In the two species, the vitelline globules, the egg shell as well as the muscle fibers were strongly positive for Bromphenol blue reaction, while other structures including the Mehlis' gland were negative. (Plate I, figs. 5 & 6; Plate II, figs. 11 & 12).

Negative results were obtained for the methyl green pyronin, Morel -Sisley diazotization and ferric chloride tests.

A summary of the results for all the tests are given in Table 1.

DISCUSSION

Egg shell formation in trematodes as exemplified in *Fasciola hepatica*[8] and adopted in textbooks is as follows: egg shell is formed by vitelline globules from the vitelline cells that coalesce at the region of the öotype and form the complete shell at the proximal uterus. This shell is made up of protein (sclerotin), phenol and phenolase.

The malachite green stain demonstrates the protein nature of the vitelline cells and globules and the egg shell. In the same manner the aldehyde fuchsin stain is a good test in determining the precursor of the egg shell. It can also indicate the keratin nature of the structure. In the larval hooks of *Öochoristica sp.* which is known to be of keratin, this test gave a positive reaction[17]. Solubility test[16,17] used on the egg shell of *C. synethes* and *G. crumenifer* with sodium sulfide solution further confirmed the keratin nature of the egg shell.

In the brown phenol blue test, the vitelline globules and egg shell were strongly positive including muscle fibers indicating that this test is not specific for egg shell material and its precursor but with other structures with basic protein.[15]

The negative results obtained in the methyl green pyronin, Morel-Sisley diazotization and ferric chloride tests indicate the absence of phenol and tyrosine. These tests show that my specimens differ from *Fasciola hepatica* in the absence of phenol and phenolase in the vitelline globules and egg shell[6,10]. It confirms the findings of Madhavi[17,18] in the absence of phenol in the vitelline globules and egg shell of the amphistomes: *Diplodiscus mehrai*, *Gastrodiscus secundus* and *Cotylophoron cotylophorum*. It is apparent therefore, that in the amphistomate egg, the basic component is protein without phenol.

As demonstrated, egg shell formation in *Camyerius synethes* and *Gastrothylax crumenifer* involve the vitelline globules, the egg shell is of the keratin type and that the Mehlis' gland does not participate in the process.

It is suggested that more histochemical and biochemical studies be made to elucidate on this hardly studied phase in the biology of these helminths.

SUMMARY

The site of the origin of the egg shell material was determined by several histochemical tests (malachite green technique, permanganate aldehyde fuchsin reaction and bromphenol blue test). Present findings show that (a) the egg shell material is formed by globules contained in the vitelline cells and (b) the egg shell material is keratin in nature, (c) the negative methyl green pyronin, Morsel-Sisley diazotization and ferric chloride tests indicate the absence of phenol and phenolase in the vitelline and egg shell of these amphistomes.

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Table 1. *Results of the Histochemical Tests*

Tests	*Carmyerius synethes*			*Gastrothylax crumenifer*		
	vitelline cells	*Mehlis' gland*	*egg shell*	*vitelline cells*	*Mehlis' gland*	*egg shell*
Malachite green	+	–	+	+	–	+
Methyl green pyronin	–	–	–	–	–	–
Bromophenol blue	++	–	++	++	–	++
Permanganate aldehyde fuchsin	++	–	++	++	–	++
Morel-Sisley diazotization	–	–	–	–	–	–
Ferric chloride	–	–	–	–	–	–

\+ – positive
++ – strongly positive
– – negative

EXPLANATION OF PLATES AND FIGURES

PLATE I – Carmycrius synethes (Fischoeder, 1901)

Fig. 1. Section of vitellaria showing positive reaction (+) to malachite green.

Fig. 2. Egg shell, positive (+) reaction to malachite green. Note vitelline globules inside egg also positive (+).

Fig. 3. Section of vitellaria showing strong positive (++) reaction to permanganate aldehyde fuchsin stain.

Fig. 4. Egg shell also strongly positive (++) to permanganate aldehyde fuchsin.

Fig. 5. Vitellaria showing strong positive (++) reaction to brom phenol blue test. Note muscle fibers also strongly positive (++) to the test.

Fig. 6. Section through Mehlis' gland and uterus containing egg. Mehlis' gland is negative (–) to brom phenol blue test while the egg shell is strongly positive (++).

PLATE II. – *Gastrothylax crumenifer* (Creplin, 1847).

Fig. 7. Section of vitellaria showing postive (+) reaction to malachite green.

Fig. 8. Egg shell also positive to malachite green.

Fig. 9. Vitelline globules showing strong positive (++) reaction to permanganate aldehyde fuchsin.

Fig. 10. Egg shell also strongly positive (++) to permanganate aldehyde fuchsin. Note strongly positive (++) vitelline globules inside egg.

Fig. 11. Vitellaria in section showing strong positive (++) reaction to brom phenol blue test.

Fig. 12. Egg shell also strongly positive (++) to brom phenol blue stain.

ABBREVIATIONS USED

egs	–	egg shell	vt	–	vitelline
mf	–	muscle fibers	vtg	–	vitelline globule
mg	–	Mehlis' gland			

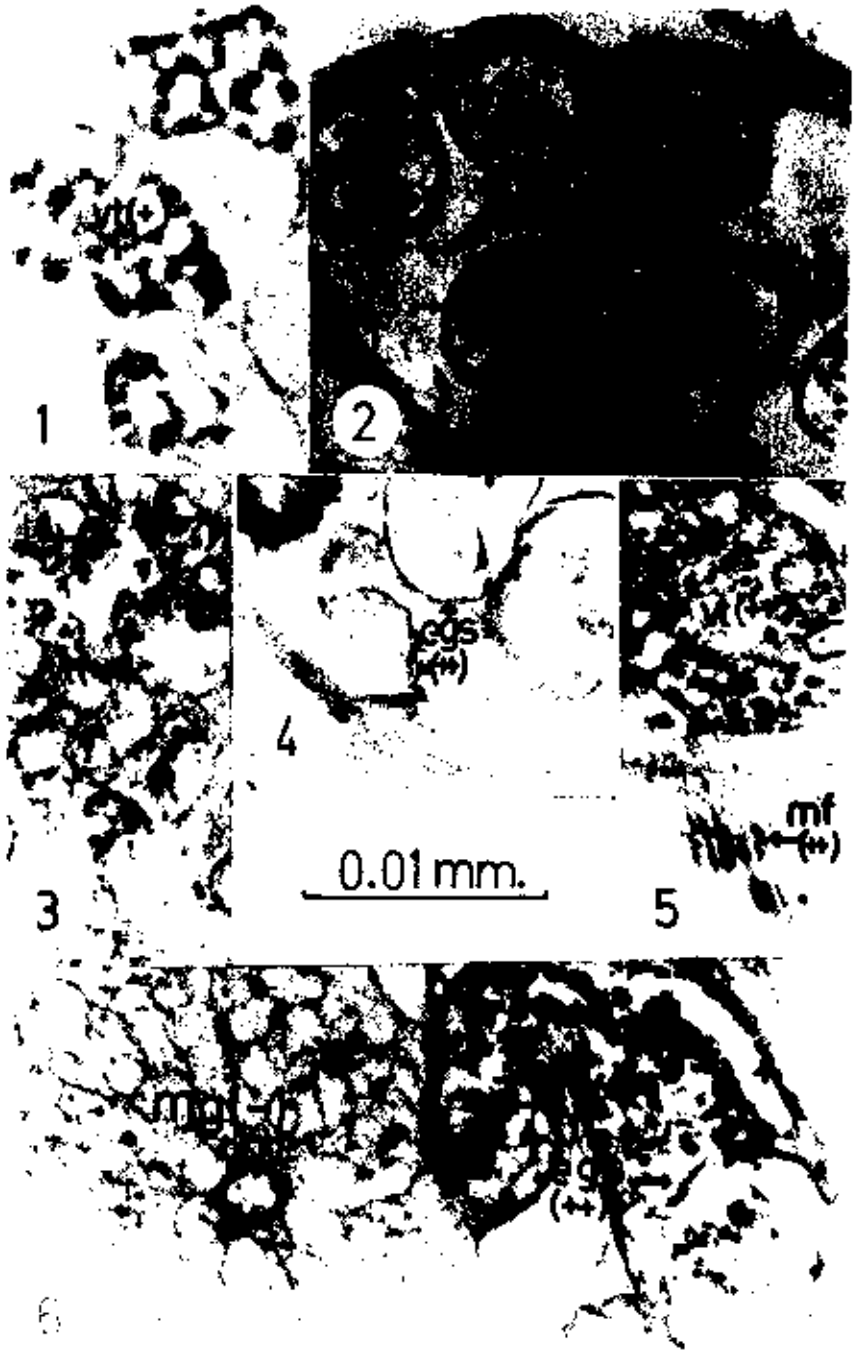

PLATE 1

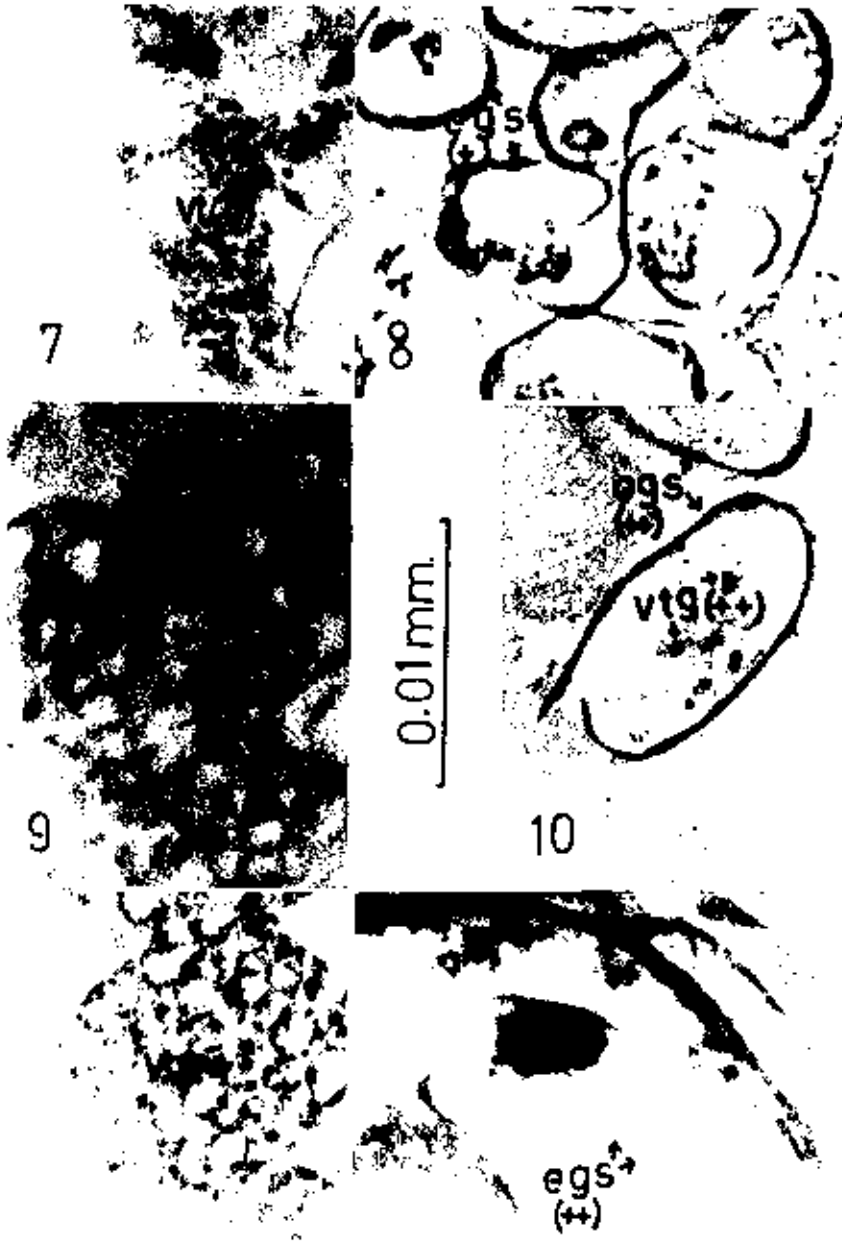

PLATE 2

THE PRODUCTION OF POTASSIUM PERMANGANATE FROM LOCAL MANGANESE ORES

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ABSTRACT

After a series of experiments, the optimum conditions under which potassium permanganate may be produced from local manganese ores was established. This involves roasting the manganese ore with 50% KOH and $KClO_3$ at a roasting temperature of 300°C, 2 hr roasting time and at a ratio of 1:3:2 for Mn ore:KOH:$KClO_3$. The calcine (K_2MnO_4) obtained is leached with hot water, allowed to stand for several hours, filtered by suction and a current of CO_2 passed through the solution. The resulting solution ($KMnO_4$) is filtered by suction again, and finally evaporated to crystalization.

INTRODUCTION

Despite the Philippines' wealth in metallic ores and industrial minerals, the development of these deposits were for several years left in the hands of foreign investors. Several years after however, a number of metallurgical projects were undertaken and others are still being planned and proposed – these projects which are believed to be basic to the national economy and to have bright prospects in the country.

With these innovations and improvements in mineral beneficiation and metallurgical processing through scientific research and development, we can now look forward to much improved and more economical processes and techniques for application on our resources.

One such natural resource is manganese. It occurs widely in crystalline rocks and like iron it is dissolved out and re-deposited in the oxide, hydroxide or carbonate form. The major ores of manganese are the oxides in hydrated

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forms and to a lesser extent the silicates and carbonates.[1] The chief oxides are pyrolusite (MnO_2), psilomelane (hydrated MnO_2) manganite ($Mn_2O_3 \cdot H_2O$), and hausmanite (Mn_3O_4).

"Pyrolusite" contains from 60-63% Mn with most of its varieties containing varying percentages of water. It is formed by the oxidation of other manganese minerals.

"Psilomelane" is an oxide of variable composition containing 45-60% manganese. It contains also a considerable amount of water.

"Manganite" $Mn_2O_3.H_2O$ contains as much as 62.4% Mn.

"Hausmanite" Mn_3O_4 contains as much as 72.1% Mn.

"Rhodochrosite" contains 47.8% manganese when pure and is the most common carbonate mineral of manganese.

The term "manganese" is usualy applied only to those ores containing more than 35% manganese. "Ferruginous manganese ores" are those containing from 10-35% manganese and those containing from 5-10% manganese are known as "manganiferous iron ores[2]". Manganese ores with less than 5% manganese with the balance mostly iron are classified as "iron ores".

From the qualitative standpoint, we have the following classification of Philippine manganese.[3]

Class	% Mn	% SiO_2	% Fe	% Al_2O_3
High grade	53.58	1.36	1.41	3.70
Marginal grade	45.14	12.86	0.96	2.79
High silica	40.81	22.46	4.48	4.19
Sub-marginal	32.64	18.69	11.39	3.35
Low-grade	20.02	14.72	13.55	11.81

It is believed that the above list fairly represents the mineralogy of the country's deposit of Mn ore. Among the important manganese districts in the Philippines are Cabadbaran, Agusan del Norte; Guindulman, Bohol; Mandaon, Masbate; Sta. Cruz, Marinduque; Siquijor; Baler, Quezon; Capas, Tarlac; Tinambac, Camarines Sur; and Busuanga, Palawan.

As of CY 1974, the Philippine manganese ore reserves were estimated at 99,378,549 M.T. containing an average of 56.9% Mn. The existence of over supply of Mn in the world, increasing competition among the large producing companies, high production costs and other unfavorable factors brought

about suspension of mining operations in the Philippines. It was revived, however, in 1976.

Manganese Ore Reserves

Location	Estimated Ore Reserves, tons	Approximate grade of ore, % Mn
Busuanga, Palawan	103,438	45.52
Guindulman, Bohol	1,600,000	28-45
Siquijor	100,000	32-52
Western Samar	43,046	32-48
Ilocos Norte	25,390	32
Baybay, Leyte	4,500	44.4
Others	97,502,175	

Manganese Ore Production
1976 and 1977

Manganese	Unit	Quantity	Value-₱
washed ore	DMT	4,302	860,488
unwashed ore	"	6,274	815,750
washed ore	"	2,151	430,222
unwashed ore	"	13,090	5,445,803

It has been estimated that about 95% of locally produced manganese is used in the metallurgical industry while the rest is used in chemical and refractory industries for the manufacture of dry batteries, disinfectants, paints and varnishes, glass decolorizer and coloring material in pottery and bricks.

Studies have shown that many chemicals with potentialities for use in industry and medicine can be extracted and derived from manganese ores, but all of these are subject to economic considerations.

Potassium permanganate which is now the object of our study is described as glittering dark, purple or bronze-like, odorless crystals, sweet, with astringent after-taste.

The uses of potassium permanganate are based exclusively on its oxidizing power. It finds application in industries and medicine as a reagent in analytical chemistry, manufacture of organic chemicals, introduction of saccharine and benzoic acid, in bleaching and decolorizing ethereal oils, waxes, and fatty materials, leather tanning, staining or preserving wood and water purification.

In medicine, it is used as topical astringent, antiseptic, disinfectant and deodorant.

Our importation of permanganates (including chromates and dichromates) as of CY 1976 totalled 176,450 kg with a value of ₱1,044,816. Most of our importations came from Italy (78,616 kg) Norway (31,000 kg), Japan (28,025 kg), China and Rep. of Taiwan (14,000 kg) and U.S. (13,415 kg) [Central Bank of the Philippines (1976)].

Our large importation of these chemicals and others had important implications on our economy. The concerted efforts of government and industry are therefore viewed with approval when these are directed to promote research and developmental projects on ores and minerals.

This project on manganese ore processing was undertaken primarily to make a study of the different methods of producing potassium permanganate and to determine the most economical and efficient method that maybe adapted to our local materials and conditions.

REVIEW OF LITERATURE

The properties of manganese described in the literature differ, showing that the impurities which characterize the metal obtained by different processes modify many of the properties. The impurities associated with manganese minerals are: 1) metals – iron, zinc, lead, copper and silver 2) gangue – silica, alumina, lime, magnesia and baria 3) volatile matter-water carbon dioxide, organic matter and 4) non-metallic – sulfur and phosphorus.[2]

If the manganese ore is to be treated economically, then silica, alumina, lime and magnesia should be kept within certain limits. To remove the water and carbon dioxide of the ore, the ore should undergo calcination. Both phosphorous and sulfur are undesirable impurities.[2].

In our present study, the first operation is ordinarily the formation of potassium manganate the second step being the oxidation of the manganate in solution to permanganate.

Chemical reactions involved:

1. $2MnO_2 + 4\,KOH + O_2 \longrightarrow 2\,K_2MnO_4 + 2\,H_2O$
2. $3K_2MnO_4 + 2\,CO_2 + H_2O \longrightarrow 2\,K_2CO_3 + 2KMnO_4 + MnO_2.2H_2O$

The most important in the process is the furnace reaction. Impurities in the ore affect the process in several ways. The presence of a small quantity of iron in the ore is beneficial, the reaction going slightly further toward

completion, the iron perhaps acting as the oxygen carrier. Silica and alumina are detrimental, passing into solution later as sodium silicate and aluminate, their subsequent removal being an occasional source of trouble. Thus the ratio of dioxide to alkali varies from 1:2 to a higher proportion of alkali, depending on the silica and alumina impurities for which additional amount must be allowed.

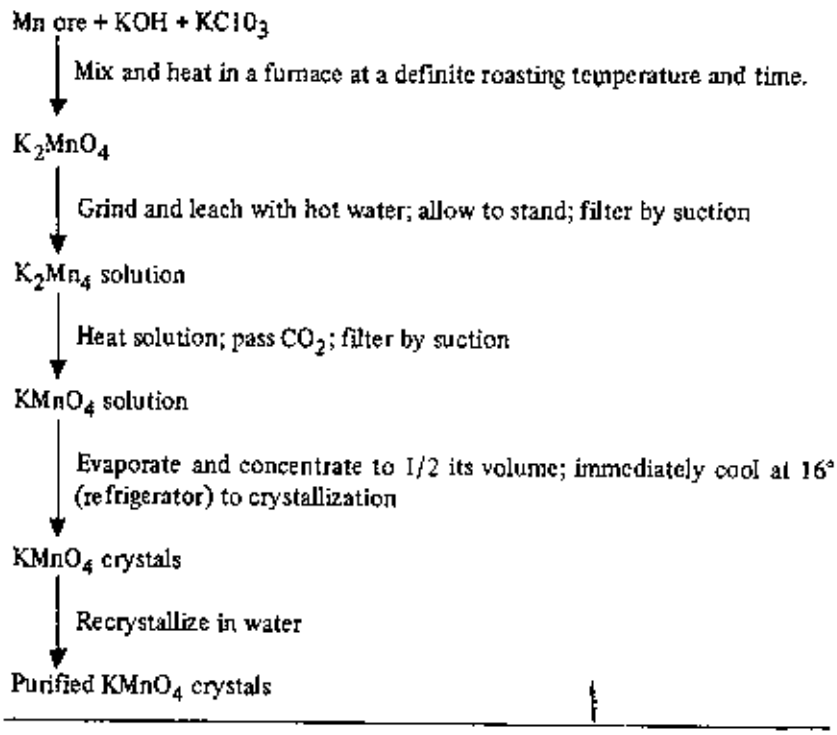
When finely powdered MnO_2 is heated with potassium hydroxide in a current of air, complete oxidation is frequently attained only by remoistening and reheating the mixture. It is heated with 50% KOH but NaOH has also been used, the $NaMnO_4.3H_2O$ being converted to the K salt as the final step of the process.

P.F. Chevillot and W.F. Edwards[4] prepared $KMnO_4$ by igniting a mixture of 1 part manganese dioxide to 1 part of 1.5 parts KNO_3, dissolving the cold product in water and evaporating the decanted red solution to crystallization. In place of KNO_3, a mixture of KOH and chlorate were recommended by G. Forchhammer, et al.[4] T.J. Pearsall[4] observed that $KMnO_4$ is formed when manganese salt is treated with chlorine and potash lye and G. Standler[4] converted manganate into permanganate by treating the solution with chlorine. P. Wagner and J. Heslinga[4] used bromines in place of chlorine to convert the manganate into permanganate.

While potassium permanganate may be obtained in several different ways as described above our studies were centered on a modified process. However, all of these methods were considered and tested for their adaptability to our local conditions and materials.

Our modified process involves the roasting of Mn ore with 50% KOH and $KClO_3$ as oxidant at a certain roasting temperature and time to produce potassium manganate (K_2MnO_4). The K_2MnO_4 produced is converted to $KMnO_4$ by leaching with hot water and passing CO_2 through the solution. The solution is filtered again by suction and evaporated on a water bath to almost 1/2 its volume and quickly cooled at 16°C (refrigerator) until crystallization takes place. The crystals are dried by vacuum. Purification of the crystals is best attained by recrystallization in water.

EXPERIMENTAL PROCEDURE


Mn ore + KOH + $KClO_3$
Mix and heat in a furnace at a definite roasting temperature and time.
K_2MnO_4
Grind and leach with hot water; allow to stand; filter by suction
K_2Mn_4 solution
Heat solution; pass CO_2; filter by suction
$KMnO_4$ solution
Evaporate and concentrate to 1/2 its volume; immediately cool at 16°C (refrigerator) to crystallization
$KMnO_4$ crystals
Recrystallize in water
Purified $KMnO_4$ crystals

Flow Diagram for the Production of $KMnO_4$

The principal raw material for permanganate manufacture is manganese ore and our study made use of manganese ore with 55.29% Mn. The manganese ores used in our experiments were heavy, hard, and titragonal crystals with an iron-black metallic color whose composition is as follows:

Mn	55.29%
SiO_2	2.39
Fe	2.0
P	.15
Al_2O_3	.28
S	.13

With this composition, our Mn ore sample which was obtained from the R.B. Industrial Company of Agusan del Norte may be classified as a natural ore of psilomelane, $MnO_2.H_2O$.

The economic production of $KMnO_4$ is very difficult because of the great care that must be taken to maintain yields and purity. It is necessary

that there be careful control in handling the reactants. Again, in the extraction step, maximum purity calls for very careful manipulation. The same problem occur throughout the process.

The manganese ore was crushed and ground and passed through a 100-mesh sieve. Fifty grams of the ground material was mixed with the theoretical amount of KOH and $KClO_3$ as oxidant. This was thoroughly mixed in a stainless steel tray and roasted in a furnace at 300°C roasting temperature and 2 hr. roasting time. After cooling the roast (K_2MnO_4), it was ground and leached with 1,500 ml of hot water. This was allowed to stand for 2 hours after which it was filtered by suction. The clear solution was heated. With an original pH of 12.7 - 13.0, CO_2 was passed through the solution until it was almost neutral. Under these conditions, 2/3 of the manganate is oxidized to potassium permanganate. The remaining 1/3, reduced to dioxide, as shown in chemical reaction below, was filtered off and mixed with ore going into the furnace.

$$3K_2MnO_4 + CO_2 \longrightarrow 2KMnO_4 + MnO_2 + 2K_2CO_3$$

The clear solution was evaporated and concentrated to 1/2 its volume, and immediately cooled in refregerator at 16°C to crystallization. The remaining liquor was decanted and the crystals dried by vacuum. Purification of the crystals was by recrystallization with water.

Thirty two grams of the $KMnO_4$ crystals were weighed and dissolved in 128 ml of water at 60°C. This was immediately filtered, evaporated and colled until crystallization took place.

Several sets of experiments were conducted to determine the type of container to use for roasting (iron pan, porcelain, casserole, and stainless steel tray); the volume of leaching water; effects of varying conditions like the amounts of KOH and $KClO_3$ (ratio: Mn ore: $KClO_3$, from 1:1:2 to 1:3:4); roasting temperature (200-500°C); and roasting period (2-4 hr).

In order to evaluate the parameters of the study, analysis of the resulting liquor and crystals obtained were made.

RESULTS AND DISCUSSION

All the methods considered and tested for their adaptability to our local conditions and materials were found to be time-consuming with non-satisfactory results. It was unwise to use chlorine since with the generations of chlorine, $KMnO_4$ would be needed. We used KOH instead of

NaOH because with NaOH, the yield is lower and the maximum conversion to manganate is found to be only 60%.

Using the modified process, the first series of experiments was conducted with the ratio of 1:2:1 (Mn ore:KOH:$KClO_3$), a roasting temperature of 300°C and a roasting period of 2 hr.

The results of a series of experiments and the conditions under which they were conducted are tabulated in Tables 1 to 5.

The first problem we encountered was on the type of container to use for roasting. Several containers were tried, namely, porcelain, casserole, clay tray (made by the Ceramics Research and Dev. Center) and a stainless steel tray. We finally decided to continue using the stainless steel tray, since the results obtained were better and a more complete reaction between the Mn ore and the KOH was observed.

The results in Table 1 show a higher yield and percent conversion of the MnO_2 to $KMnO_4$ by using 1,500 ml H_2O for leaching. Note that there was a corresponding decrease of yield as we increased the volume of water.

In Table 2, it is evident that at 200°C, poor results were obtained, But as we raised the temperature to 300°C, better results were obtained. However, it will be noted that as the temperature was further increased there was a corresponding decrease of the yield and percent conversion to $KMnO_4$.

In Table 3, better results were attained with a roasting time of 3 hr.

In Table 4, the best results were attained with the ratios, 1:2:3 and 1:3:2 at roasting times of 3 and 2 hr, respectively.

To further determine which of the above conditions would give the best results with respect to purity of crystals, experiments were carried out up to the crystallization and purification of the crystals.

Table 5 shows that the best results were obtained based on the highest yield and purity of crystals, when the mixture of 1 part Mn ore to 3 parts of KOH and 2 parts of $KClO_3$ were roasted for 2 hr at a roasting temperature of 300°C. In Table 6 a comparison is made of the ratios 1:2:3 and 1:3:2 (Mn ore:KOH:$KClO_3$).

Several verification studies were also conducted using the above conditions. Prelimanary cost estimate of the production of $KMnO_4$ (laboratory scale) indicated that under the optimum conditions 1:3:2 ratio

(Mn ore:KOH:$KClO_3$), roasting temperature, 300°C and roasting time, 2 hr, the best crystals could be produced at a much lower cost.

Based on these results using the ratio 1:3:2 (Mn ore:KOH:$KClO_3$), the ratio of equivalent of Mn content (of the ore) to the KOH solution (50%) and to the $KClO_3$ would be 1:3.87:.4695 as explained in the computation:

50 g Mn ore (55.29%) — 214.02 ml KOH (50%)

Mn ore: KOH:$KClO_3$

$$\underline{1} : \underline{3} : \underline{2}$$

$$\frac{214.02}{55.29} = 3.87$$

50 g Mn ore — 25.96 g $KClO_3$

Mn ore:KOH:$KClO_3$

$$\underline{1} : \underline{3} : \underline{2}$$

$$\frac{25.96}{55.29} = 0.4695$$

From the above ratio constants 3.87 (KOH) and 0.4695 ($KClO_3$) were derived, which if multiplied by g manganese in the ore would give the number of ml of KOH solution and g of $KClO_3$ that would be roasted with it, thus 55.29 x 3.87 = 214.02 ml KOH (50%) and 55.29 x 0.4695 = 25.96 g $KClO_3$. So a manganese ore sample containing 55.29% Mn would be using 214 ml KOH (50%) and 25.96 g $KClO_3$ per 50 g manganese ore. The optimum conditions now established are:

Roasting temperature	–	300°C
Roasting time	–	2 hr
Ratio of Mn:ore:KOH:$KClO_3$	–	1:3.87:0.4695

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Table 1. *Effect of Varying Volume of Leaching Water on the Conversion of MnO_2 to $KMno_4$*

Roasting temperature	300°C	
Roasting time	2 hr	
Mn ore: KOH:$KC10_3$ ratio	1:2:1	

Vol. of H_2O, ml	g $KMnO_4$/50 g ore	$KMnO_4$, %	Conversion to $KMnO_4$, %
1,000	17.95	37.29	44.65
1,500	20.85	41.36	52.0
2,000	18.57	39.49	46.1
3,000	16.17	37.52	41.0

Table 2. *Effect of Varying Roasting Temperature on the Conversion of MnO_2 to $KMnO_4$*

Roasting time	2 hr
Ratio (Mn ore:KOH:$KClO_3$)	1:2:1
Volume of leaching water	1,500 ml

Roasting temp., °C	g $KMnO_4$/50 g ore	$KMnO_4$, %	Conversion to $KMnO_4$, %
200	15.14	30.26	37.97
300	20.85	41.36	52.0
400	16.88	33.77	42.19
500	13.91	26.2	31.0

Table 3. *Effect of Roasting Time on the Conversion of MnO_2 $KMnO_4$*

Roasting temperature	300°C
Ratio (Mn ore:KOH:$KC10_3$)	1:2:1
Volume of leaching water	1,500 ml

Roasting time, hr	g $KMnO_4$/50 g ore	$KMnO_4$, %	Conversion to $KMnO_4$, %
2	20.85	41.36	52.0
3	26.56	52.47	65.58
4	17.38	34.82	43.45

Table 4. *Effect of Varying Ratio of Mn Ore to $KClO_3$ and Time of Roasting on the Conversion of MnO_2 to $KMnO_4$*

Roasting temperature		300°C		
Volume of leaching water		1,500 ml		
Ratio (Mn ore: KOH: $KClO_3$)	Roasting time, hr	g $KMnO_4$/50 g ore	$KMnO_4$, %	Conversion to $KMnO_4$, %
	2	20.85	41.36	52.0
1:2:1	3	21.56	42.46	53.07
	2	17.30	34.61	43.26
1:2:2	3	19.86	41.21	49.65
	2	29.72	59.47	74.30
1:2:3*	3*	32.33	64.73	80.83
	2	28.08	56.17	70.20
1:3:1	3	29.16	58.34	72.91
	2*	32.08	64.28	80.27
1:3:2*	3	28.51	57.15	71.27
	2	23.9	47.14	59.05
1:3:3	3	25.84	51.92	64.59
	2	19.15	38.27	47.87
1:3:4	3	27.02	53.74	67.55

Table 5. *Best Results Obtained Based on the Highest Yield and Purity of Crystals*

Roasting Temperature		300°C					
Leaching water (ml)		1,500 ml					
Ratio (Mn ore: KOH:$KClO_3$)	Roasting time, hr	g $KMnO_4$ 50 g ore	$KmnO_4$ %	Convertion to $KMnO_4$ %	Crude %	Purity 1st Recryst.	Purity 2nd Recryst.
1:2:1	2	17.35	34.7	43.37	54.08	61.26	81.0
	3	21.56	42.46	53.07	56.0	73.51	98.08
1:2:2	2	17.30	34.61	43.26	37.27	52.99	70.2
	3	19.86	41.21	49.65	45.09	75.74	85.0
1:2:3	2	29.72	59.47	74.30	52.55	65.81	85.95
	3	32.33	64.73	80.83	60.92	81.96	97.68
1:3:1	2	28.08	53.13	70.20	75.58	96.0	–
	3	29.16	58.34	72.91	81.86	96.04	–
1:3:2*	2*	32.08	64.28	80.27	82.24	96.01	98.06-99.92
	3	28.51	57.15	71.27	81.78	93.0	96.0
1:3:3	2	23.29	47.14	59.05	69.79	90.0	–
	3	25.84	51.92	64.59	68.17	–	–
1:3:4	2	19.15	38.27	47.87	28.64	–	–
	3	27.02	53.74	67.55	20.11	–	–

Table 6. *A Comparison of Results Obtained from Ratios 1:2:3 and 1:3:2 (Mn ore:KOH:$KClO_3$)*

Roasting temperature		300°C	
Volume of leaching water		1,500 ml	

Ratio (Mn ore: KOH:$KClO_3$)	Roasting time, hr	Purity, % (crude crystals	1st Recryst.	Purity, % 2nd Recryst.
1:2:3	3	60.92	81.96	97.68
1:3:2*	2	82.24	96.01	98.06-99.92

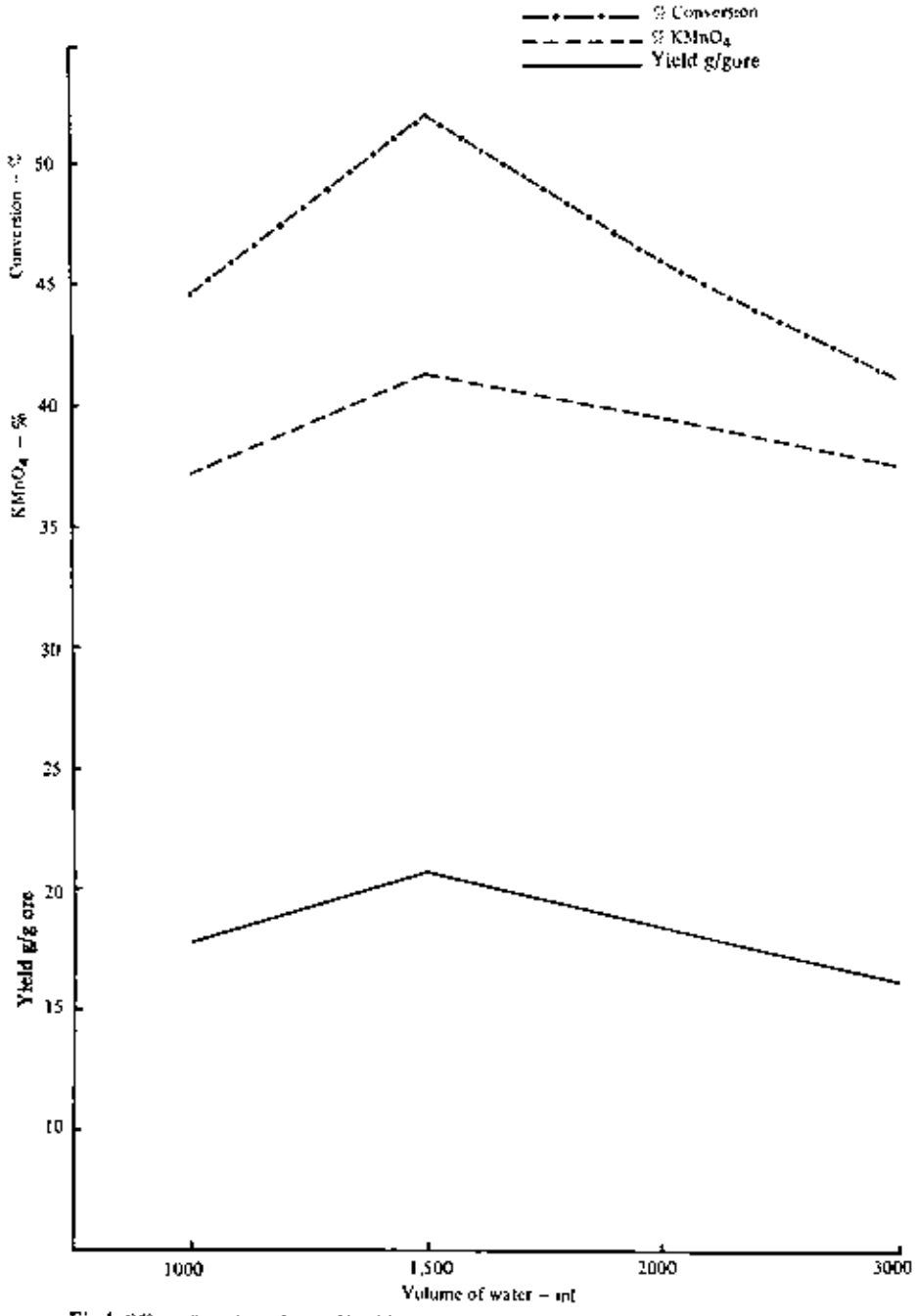

Fig. 1 Effect of varying volume of leaching water

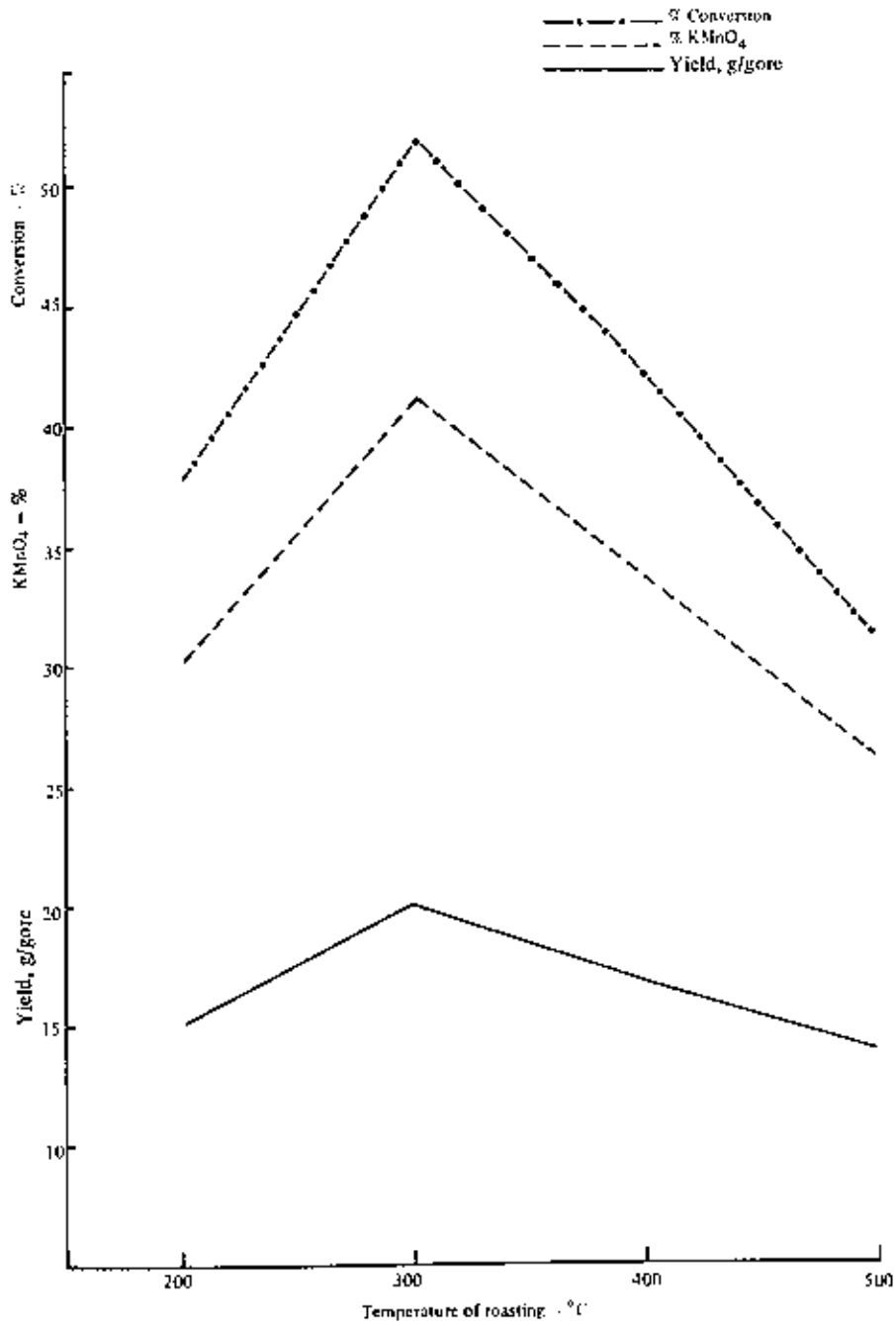

Fig. 2 Effect of roasting temperature

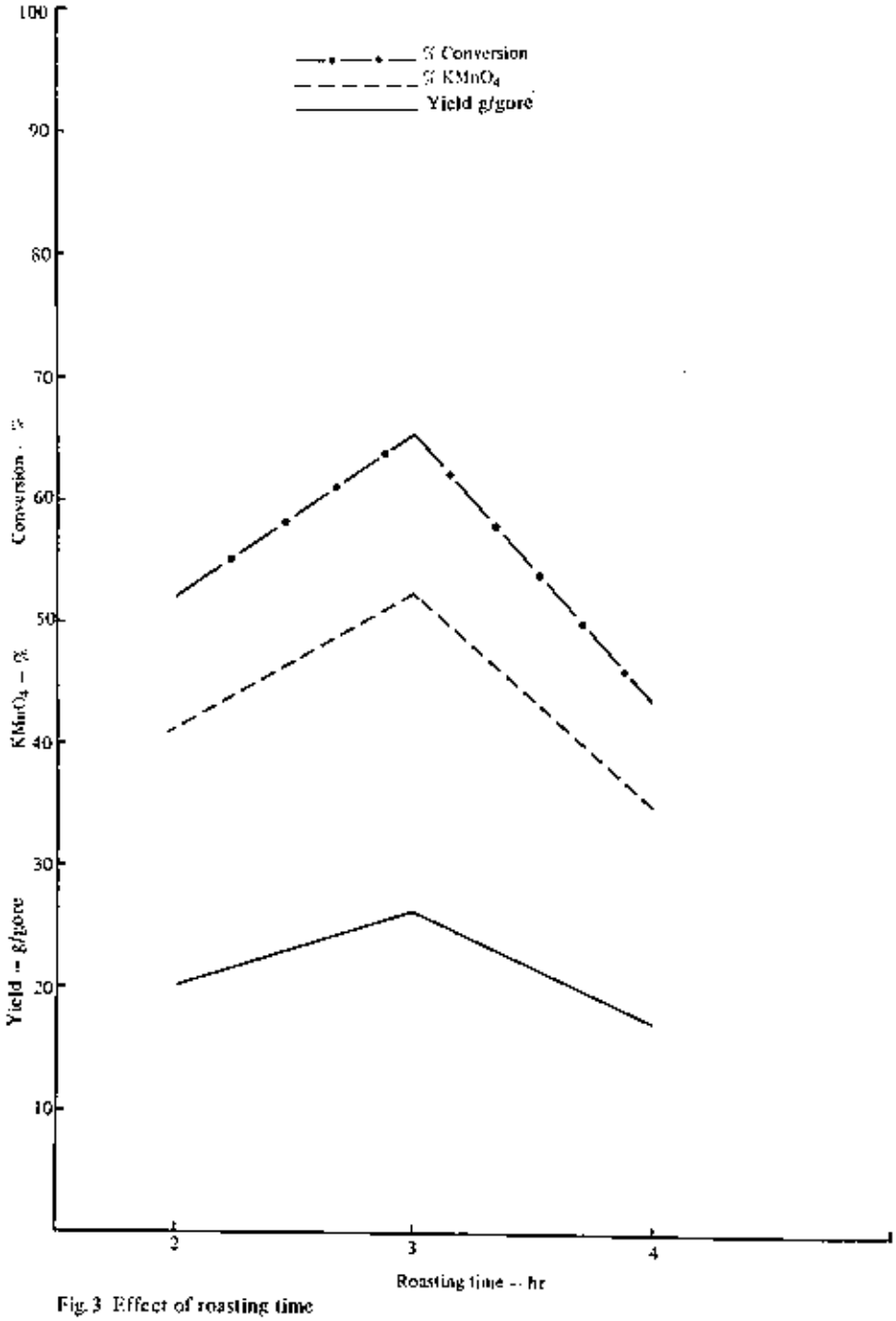

Fig. 3 Effect of roasting time

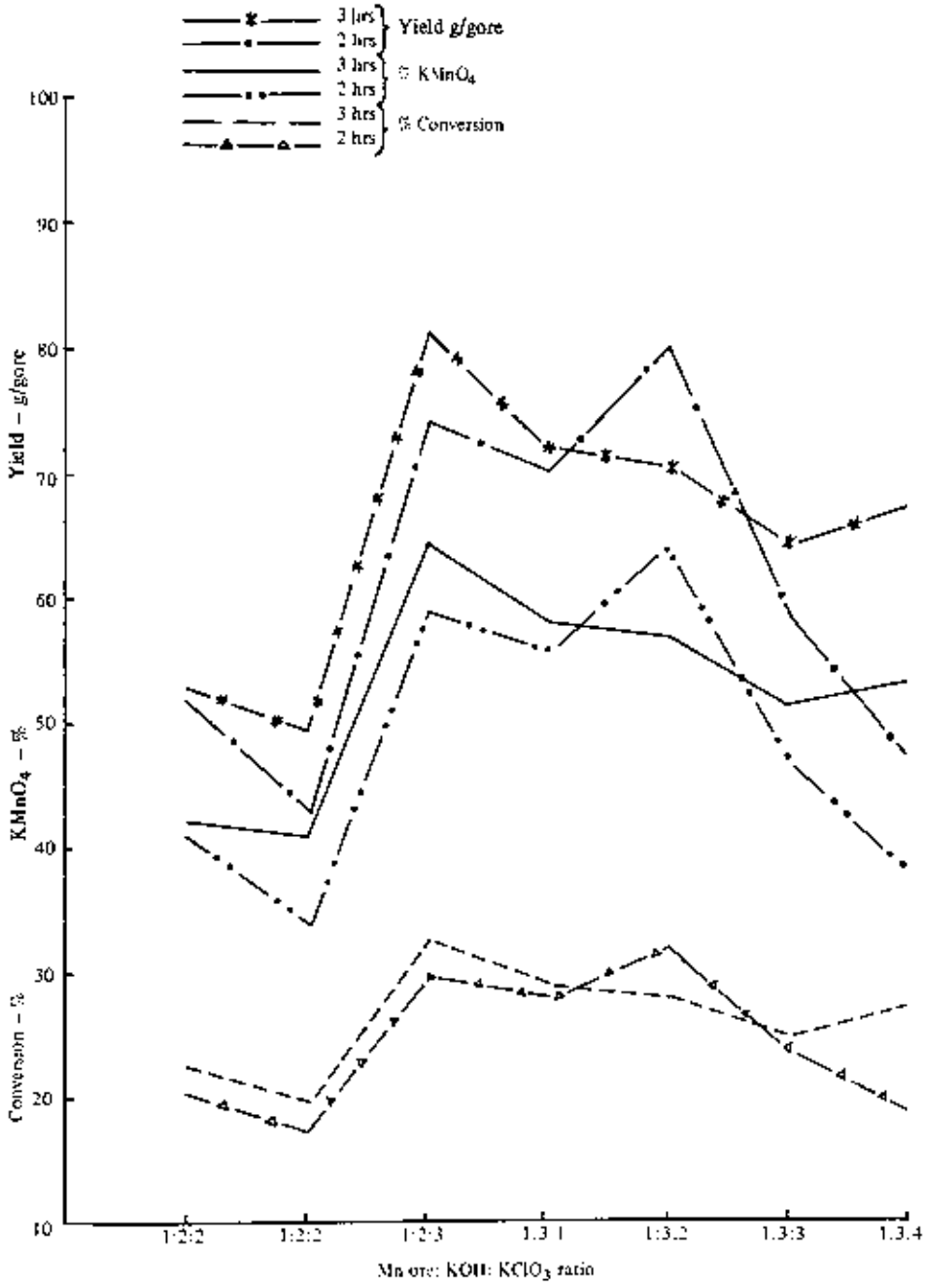

Fig. 4 Effect of varying ratio of Mn ore: KOH: $KClO_3$

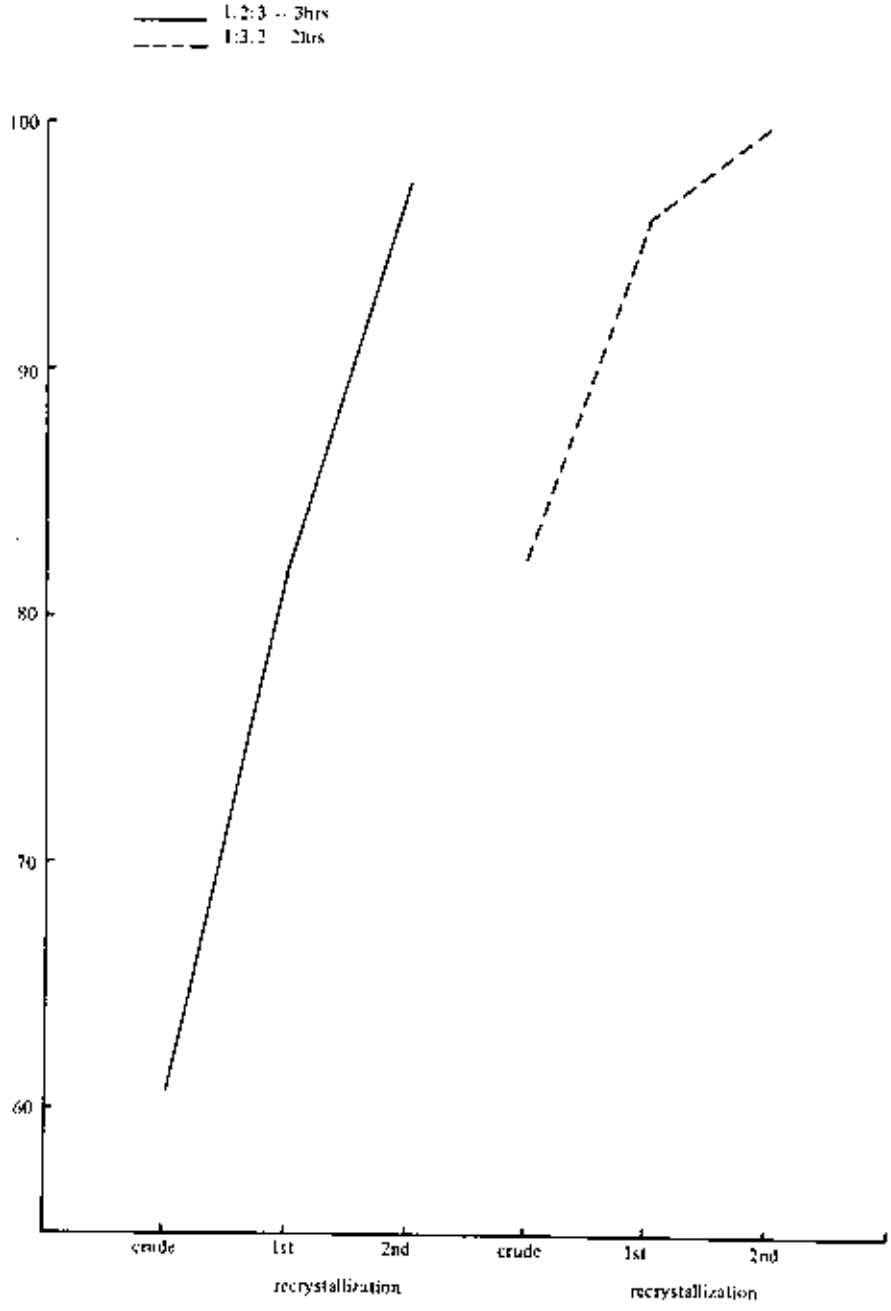

Fig. 5 Comparison of the purity of crystals

MOSSES NEW TO THE PHILIPPINES[1]

R. M. DEL ROSARIO[2]

and

B. O. VAN ZANTEN[3]

ABSTRACT

Eleven mosses are reported new to the Philippines. The collections were made from 1,000-2,880 m in the Cordillera Central, Luzon, which is the most extensive system of highland areas in the Philippines.

In November 1968 and from 1973-1976 at different times of each extensive bryological collections were made in the Cordillera Central, a mountainous range that lies in the Northern part of Luzon, formerly known in Philippine geography as Mountain Province. It is in the Cordillera Central that the highest point is reached (Mt. Pulog, 2,880 m). Only Mt. Apo in Mindanao is higher than Mt. Pulog, reaching 2,930 m. Farther north, about 10 kms away from Mt. Pulog, is the second highest peak in Luzon, (Mt. Tabayoc, 2,819 m). The other high summits in the Cordillera Central, Luzon are Mt. Panotoan, with two peaks (2,650 and 2,645 m) lying between Mt. Pulog and Mt. Tabayoc, Mt. Data (2,300 m), and Mt. Santo Tomas (2,258 m).

As most of the Luzon Cordillera Central lies above 1,000 m, it forms a unique climatic and biogeographic region. The cold-loving vegetation of the region was studied earlier by Merill and Merritt[1] and recently by Jacobs[2] who

[1]The first author was supported by grants from the National Research Council of the Philippines and the Technical Assistance Department of Netherlands Ministry of Foreign Affairs. The second author's collecting trip in 1968 was financed by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO). Dr. M. Jacobs' collections were made available by Dr. A. Touw, Rijksherbarium, Leiden, to whom apreciation is extended. The illustrations were prepared with skill and understanding by Mr. Severino C. Reyes under NRCP Project I.E-34. Special thanks are also extended to Mr. Gerard L. Penecilla of the University of the Philippines–Natural Science Research Center for his assistance in many ways.

[2]National Museum of the Philippines, Rizal Park, Manila Philippines.

[3]Biologisch Centrum, Plantensystematiek, Haren, (Gr.), The Netherlands.

made an extensive general collection during his 1968 exploration. It is interesting to know that he submitted to Philippine authorities a formal proposal for the conservation of Luzon's highest mountains.

The specimens cited here are deposited at the Philippine National Herbarium, and Biological Center of the State University of Groningen in Haren, The Netherlands. The species reported in this paper are arranged alphabetically. Since literature regarding these species in the Philippines is scarce and hardly available, each species is again fully illustrated even if they have been included and illustrated in some other countries' moss flora. The main bulk of the collections will comprise the material for the preparation of a Handbook of Philippine Mosses together with those of the central and southern part of the country where further exploration will commence early part of 1978.

Campylopus polytrichoides De Not. On soil among grasses and growing with *Aongstroemia orientalis*, Mt. Polog, summit, R.M. del Rosario *15607*. — The outstanding characteristics of this species are the presence of low dorsal lamellae and the lax basal cells. It differs from *C. introflexus* by its straight leaf tip and narrower leaves. This species is occurring in India, Ceylon, Java, Sumatra, Africa, south and western Europe.

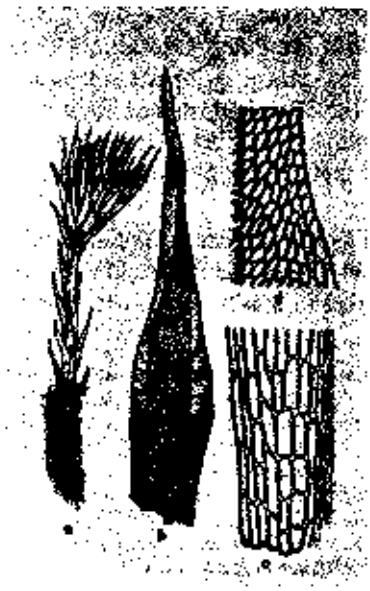

PLATE (1) *Campylopus polytrichoides* De Not.

a. Plant habit – X 10
b. Leaf – X 50
c. Basal leaf Cells – X 360
d. Median leaf cells – X 360

Dicranoloma billardieri (Brid.) Par. On bark of logs, Mt. Data, summit; R.M. del Rosario *15605*. This species differs from the other local species by its less setaceous subula which is more or less flattened above and the dentation which is reduced or even absent in many cases. The species occurs in the temperate climates of the Southern Hemisphere.

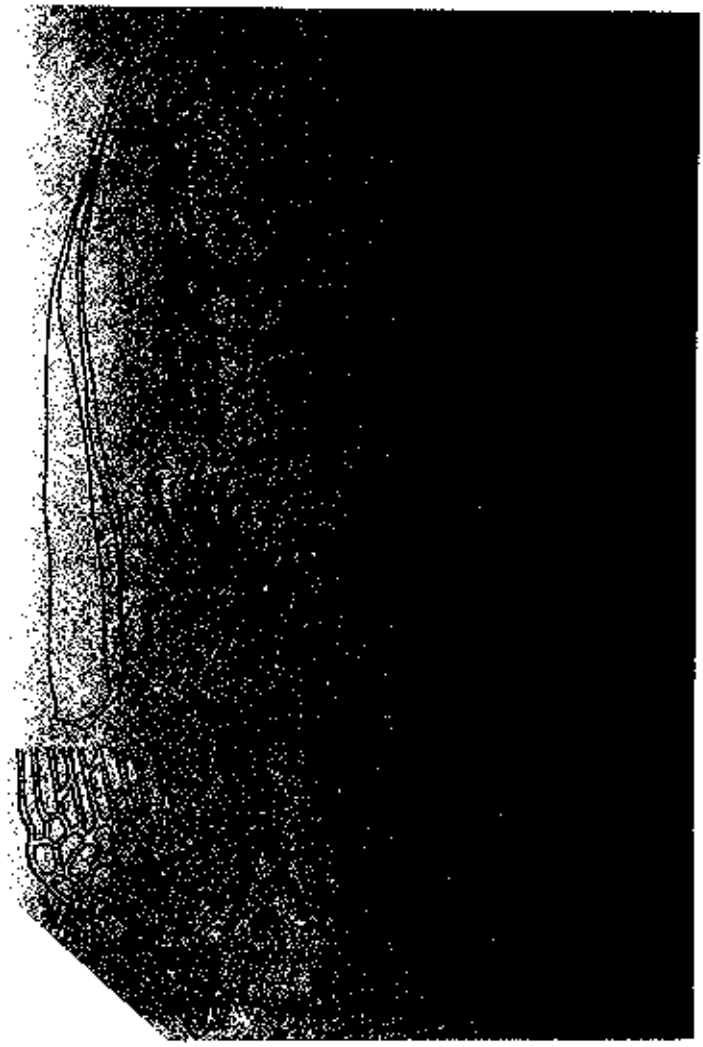

PLATE (2) *Dicranoloma billardieri* (Brid.) Par.

a. Plant habit – X 9
b_1 & b_2 Leaves – X 29
c. Basal leaf cells X190
d. Median leaf cells – X 190
e. Apical leaf cells – X190

Dixonia thamnioides (Broth. et Dix.) Bosik. et Ando. Mt. Tabayoc, Jacobs ***BS49*** (determined by A. Touw.). – The distinctive characteristics of this species are the more or less dendroid habit with flagelliform branches, oval complanate leaves, indistinct double costa, smooth linear leaf cells, and denticulate upper leaf margins. The species has been reported from Ceylon and Thailand.

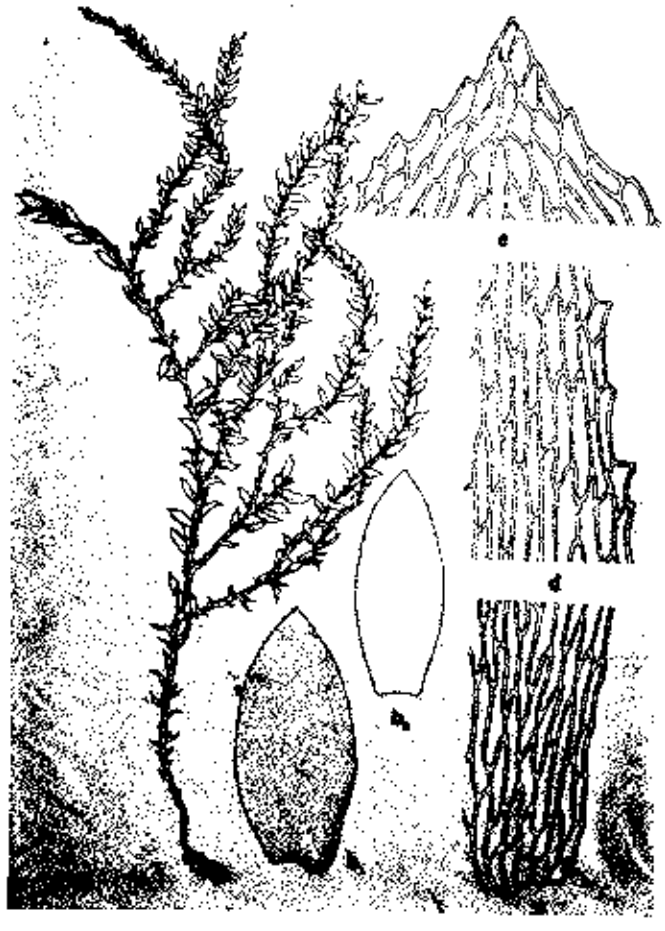

PLATE (3) *Dixonia thamnioides* (Broth. et Dix.) Bosik. et Ando.

a. Plant habit – X 5
b1 & b2 Leaves – X 50
c. Basal leaf cells – X 330
d. Median leaf cells – X 330
e. Apical leaf cells – X 330

Fissidens asplenioides Hedw. Mt. Polis, on rock crevices, B.O. van Zanten *68-11-4029*; Mt. Santo Tomas, R.M. del Rosario *7296A*. It is distinguished from its allied species by the obtuse leaf apex which is usually circinate when dry. The species has a worldwide distribution. It was collected in New Guinea for the first time by; B.O. van Zanten[4] *10-68-809; 10-68-2936B; 11-68-3632*.

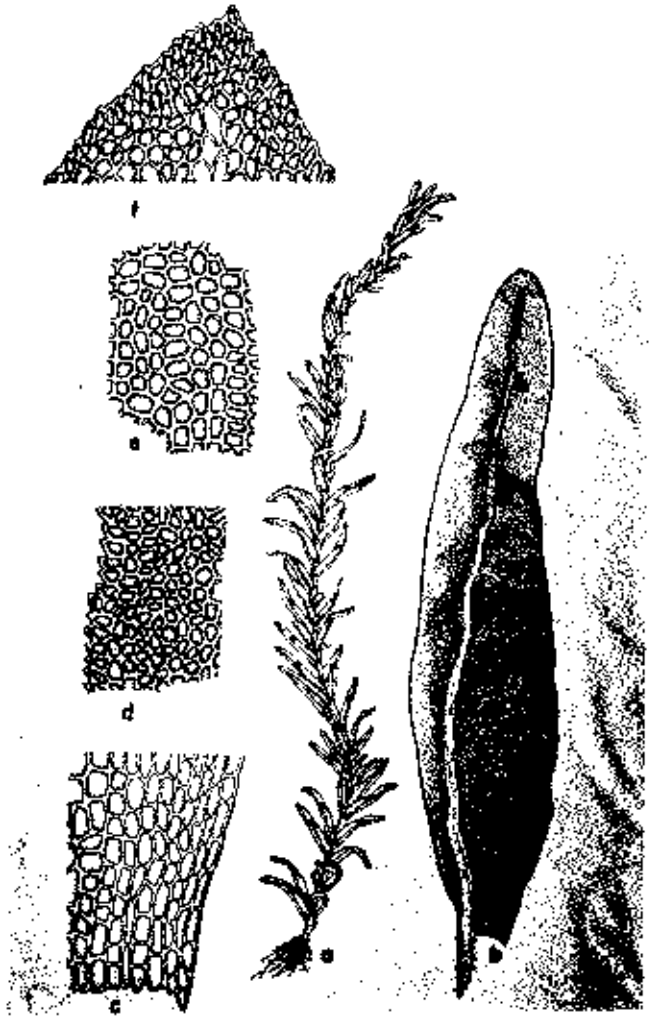

PLATE (4) *Fissidens asplenioides* Hedw.

a. Plant habit – X 6.5
b. Leaf – X 34
c. Basal leaf cells – X 320
d. Medium leaf cells – X 320
e. Leaf cells of the dorsal blade – X 320
f. Apical leaf cells – X 320

Floribundaria sparsa (Mitt.) Broth. var. *pilifera* (Nog.) Nog. Mt. Tabayoc, on trunks, R.M. del Rosario *14698*. Noguchi[5] separates this species from *Floribundaria aurea* by its yellowish to deep green habit, the latter are yellowish-green to reddish-brown. The branch leaves are scarcely conplanate and the marginal teeth rather small.

PLATE (5) *Floribundaria sparsa* (Mitt.) Broth. var. *pilifera* (Nog.) Nog.

a. Plant habit – X 9
b. Leaf – X 90
c. Median leaf cells – X 330
d. Basal leaf cells – X 330

Meteoriella soluta (Mitt.) Okam. Mt. Tabayoc, Jacobs *B651 (determined by* A. Touw.). This species can be easily separated from other local Meteoriaceae by its very faint to indistinct double costa, orange colored leaf base with large auricles, and the smooth very thick-walled linear cells. The leaf has a broadly ovate base becoming abruptly acuminate. India, China, Taiwan, Japan, Philippines.

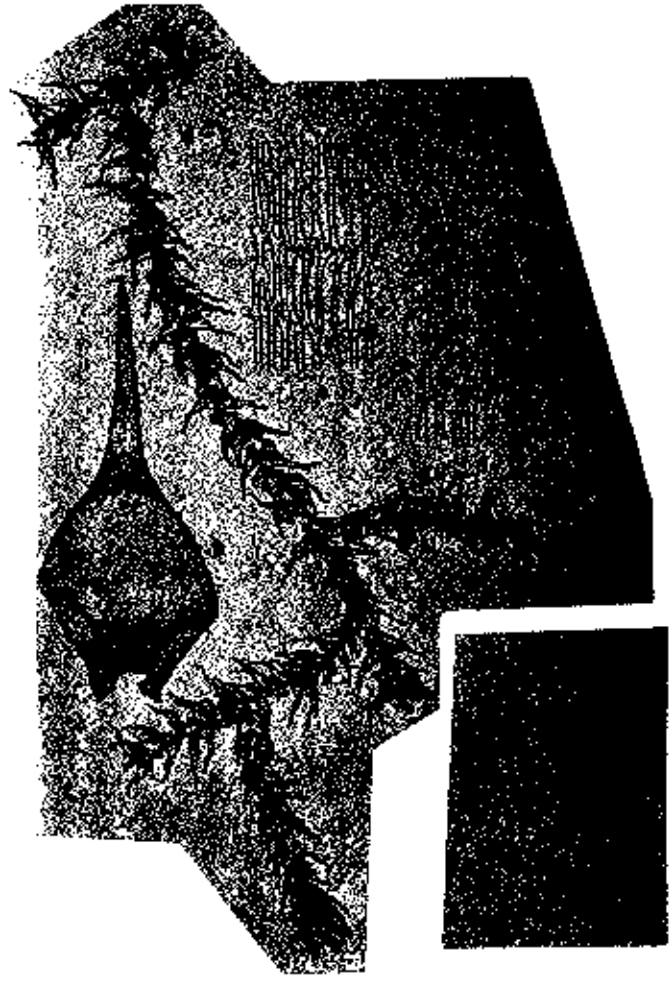

PLATE (6) *Meteoriella soluta* (Mitt.) Okam.

a. Plant habit – X 9
b. Leaf – X 32
c. Basal leaf cells – X 330
d. Apical leaf cells – X 330
e. Median leaf cells – X 330

Microcampylopus subnanus (C. Muell.) Fleisch. Mt. Santo Tomas, on soil, R.M. del Rosario *7515*. Because we could not find stomata on the bases of the capsules which we examined, we place the plants under *Microcampylopus*. Otherwise, they look similar to *Campylopodium euphoroeladum*, except for the narrower leaves. This species is also found in Java.

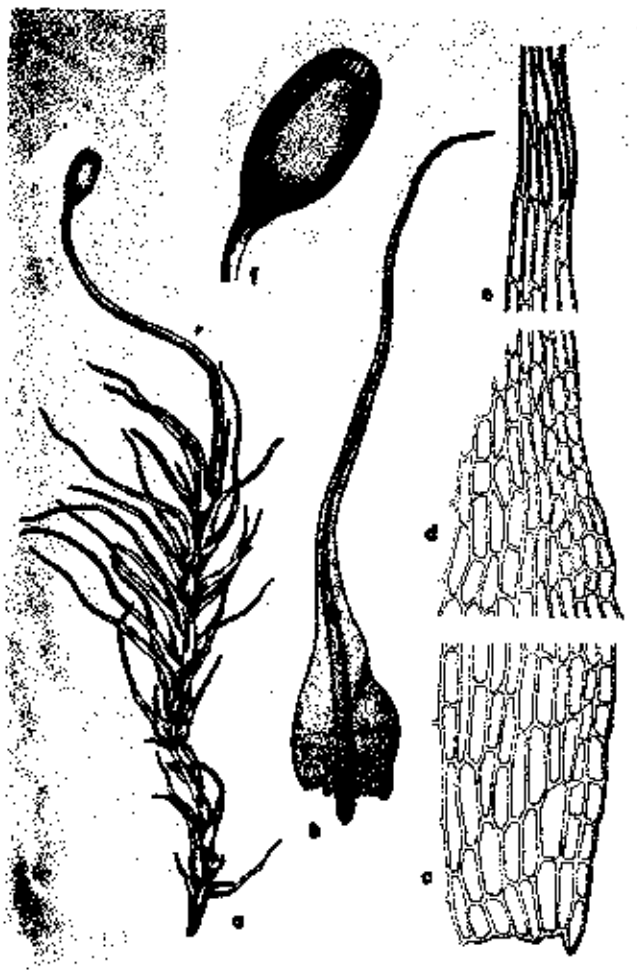

PLATE (7) *Microcampylopus subnanus* (C. Muell.) Fleisch.

a. Plant habit – X 13
b. Leaf – X 48
c. Basal Leaf cells – X 330
d. Median leaf cells – X 330
e. Upper leaf cells – X 330
f. Capsule – X 49

Mnium laevinerve Card. Mt. Puiog, on boulders in creeks, R.M. del Rosario *12014, 12040, 12043, 12075b, 12076* (determined by Koponen). Koponen[6] states that it is mainly temperate, growing from the southern temperate to the northern temperate zone but it extends its range up to the middle orohoreal zone. While the typical habitats are creek banks and cliff faces, the plants grow also on rotten wood.

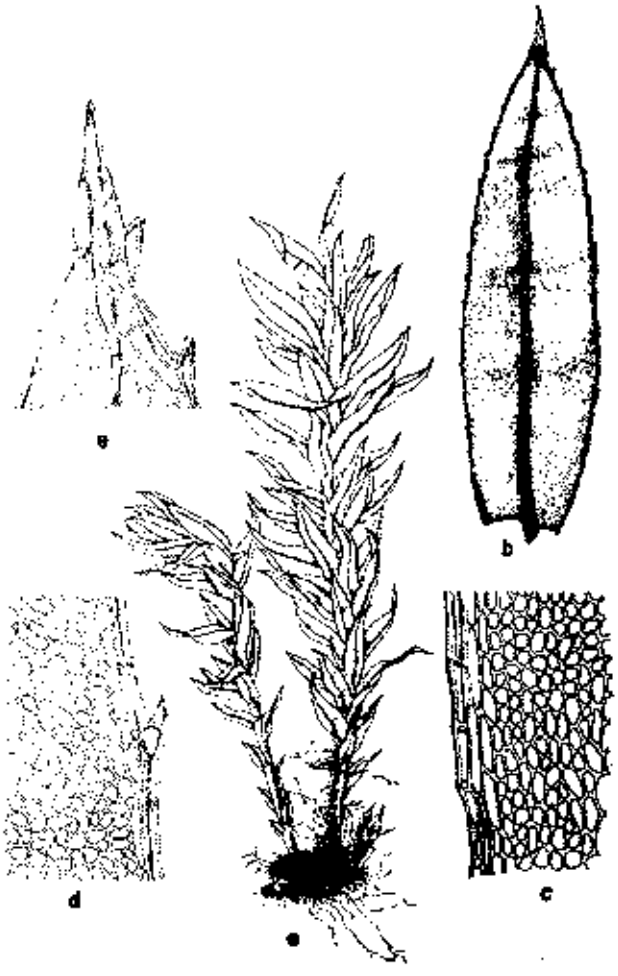

PLATE 181 *Mnium laevinerve* Card.
a. Plant habit – X 6
b. Leaf – X 25
c. Basal leaf cells – X 210
d. Median leaf cells – X 210
e. Apical leaf cells – X 210

Platyhypnidium wildei Zant. Imelda Park, Baguio; on stone walls, R.M. del Rosario *7039*. – The specimens agree well with the type material from New Guinea. The species differs from *P. muelleri* by its more acuminate leaf with the acumen being often twisted and the upper lamina cells are shorter and rhomboidal, ranging from 16-30 µm. In *P. wildei*, the upper lamina cells range from 35-70 µm. This species differs from *Rhynchostegium asperisetum* by the sharply serrulate branch leaf margins. The species was formerly known from a collection in the Star Mountains, West New Guinea; van Zanten[4].

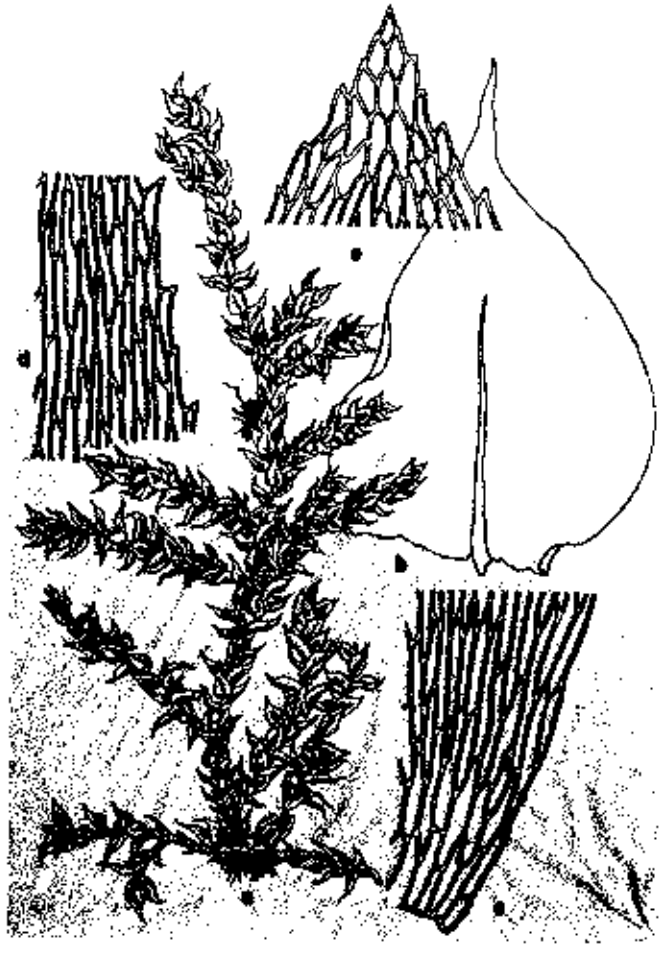

PLATE (9) *Platyhypnidium wildei* Zant.

a. Plant habit – X 6.4
b. Leaf – X 50
c. Basal leaf cells - X 320
d. Median leaf cells – X 320
e. Apical leaf cells – X 320

Syrrhopodon treubii Fleisch. Mt. Pulog, on trunks; R.M. del Rosario *12089, 12090;* Mt. Tabayoc, R.M. del Rosario *14013.* – This species differs distinctly from *S. gardneri* by its cancellinae passing gradually into the lamina cells, whereas the marginal cells of the leaf base which are usually longer and narrower abruptly extending to the cancellinae. *Calymperes datense* might prove to be conspecific with the present species but that because we have not seen its type material we could not make the reduction possible at this time.

PLATE (10) *Syrrhopodon treubii* Fleisch.

a. Plant habit – X 6
b. Leaf – X 30
c. Basal Leaf cells – X 210
d. Median leaf cells – X 210
e. Upper leaf cells – X 210
f. Apical leaf cells – X 210

Warburgiella subleptorrhynchoides (Fleisch.) Fleisch. Mt. Pulog, on barks of trees, R.M. del Rosario *12038;* Santo Tomas, R.M. del Rosario *7383B.* The species may easily be confused with *W. cupressinoides* but the latter has the leaves tapering gradually to a filiform point while in the former the leaves taper abruptly. The two species are very similar in size and appearance. Both are larger and less delicate than *W. philippinensis* which is more closely similar to *W. cupressinoides.*

PLATE (11) *Warburgiella subleptorrhynchoides* (Fleisch.) Fleisch.

a. Plant habit – X 7
b_1 & b_2 Leaves – X 90
c. Basal leaf cells – X 330
d. Median leaf cells – X 330
e. Apical leaf cells – X 330

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GENETICS OF DIELDRIN RESISTANCE IN *CULEX TRITAENIORHYNCHUS* – A PRELIMINARY REPORT

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ABSTRACT

Dieldrin resistance allele (D1) was isolated from two wild type stocks of *Culex tritaeniorhynchus* and homozygous colonies laboratory-bred. Larval mortality tests with different concentrations of dieldrin reveal that 0.125 PPM is the best discriminating dose in which all the +/+ larvae die in the first two hours, the D1/+ die between 2¼ to 8 hours, while D1/D1 remain alive 24 hours after. The data obtained from different genetic crosses indicate that D1 is an incompletely dominant allele, located on the submetacentric chromosome (linkage group II) at a distance of 26.46 ± 1.26 map units from red-potted eye, another autosomal codominant allele.

INTRODUCTION

Insecticides and pesticides were once considered to be the most effective weapon in man's struggle against harmful insects. However, the limitations of these chemicals were soon realized by workers in the laboratory and field. The occurrence of genetic plasticity in natural insect populations and subsequent development of resistant strains against these chemicals served to obviate the latter's usefulness as control agents.

Studies have been done to find the effective minimum dose of these chemicals as well as resistant strains in natural populations of mosquitoes. However, only a few of these deal with inheritance patterns. The genetics of dieldrin resistance has been worked out by French and Kitzmiller[1] in *Anopheles quadrimaculatus* and by Lockhart[2] *et al* in *Aedes aegypti*. In both mosquito species dieldrin resistance is inherited as a single autosomal allele, showing incomplete dominance over its normal counterpart.

The present study on *Culex tritaeniorhynchus* Giles, an important vector of Japanese #B# encephalitis and West Nile viruses in Asia, was undertaken for two reasons: firstly, to isolate a good marker gene, which can be used in genetic linkage studies; and secondly, to determine the minimum

* To who inquiries are to be made.

effective dose of dieldrin, which can be used in a well coordinated control programme. This paper reports the methods of selection of dieldrin-resistance gene and preliminary data on its mode of inheritance in *C. tritaeniorhynchus*.

METHODS AND MATERIALS

I. Isolation and Selection of Dieldrin-resistance Gene:

Two basic wild-type stocks of *C. tritaeniorhynchus* Ravi bank and Khulna were choosen for isolation and selection of dieldrin-resistance mutant (D1). The stocks are being maintained at the Pakistan Medical Research Center in Lahore after Baker and Aslamkhan.[3] The techniques used for mosquito reading were similar to Baker [4] *et al.* The methods adapted for the isolation and selection of D1 were similar to French and Kitzmiller[1] with some modifications.

Well-fed fourth instar larvae from the aforementioned wild-type stocks were exposed to 2.5 PPM (parts per million) of dieldrin for three hours. The few survivors of this larval treatment were taken out and washed with running tap water. Every care was taken to save the larvae from possible injury during this handling. These larvae were allowed to develop into adults, which were then interbred. The larvae obtained repeated generation after generations until a vigorously reproducing stock was produced.

Once a potential resistant stock was produced, it was desired to obtain a minimum dose of dieldrin which can discriminate between the sensitive and the resistant larvae. For this purpose, batches of 25 larvae of both potential resistant and normal Ravi bank stocks were exposed to 0.00, 0.02, 0.1 0.5 and 2.5 PPM of dieldrin and larval mortality recorded every hour. The experiment was run in duplicate and each experiment done twice to minimize error due to chance. The result of this experiment revealed that 0.1 PPM dieldrin is a selective dose which can kill all sensitive larvae in the first three hours while 20% of the potential resistant stocks can tolerate this dose even for 24 hours. Further selection of the potential resistant stock was carried at 0.1 PPM dieldrin, used for 24 hours. Our observations showed that the survivors of this treatment produced equally resistant progeny. Thus a homogeneous stock of homozygous dieldrin-resistant individuals was produced.

II. Selection of a Suitable Dieldrin Dose for Genetic Studies:

The genetic studies on dieldrin-resistance required a dose of dieldrin which can differentiate between the three F_2 genotypes i.e. +/+; D1/+ and D1/D1.

For this purpose, batches of 25 well-fed larvae from homozygous sensitive (Ravi bank); F_1 sensitige X resistant and homozygous resistant stocks were exposed to 0.02,0.05,0.075,0.100,0.125,0.0.150 and 0.175 PPM dieldrin and larval mortality recorded every 15 minutes for the first three hours and every hour thereafter. The experiment was run in duplicates and done twice. The results of this experiment showed that 0.125 is the best discriminating dose, where all the +/+ larvae die in first two hours, while D 1/+ show progressive mortality from 2¾ hours to 8 hours. The D1/D1 group remain alive after 24 hours.

III. Genetic Experimentation:

The following strains of *C. tritaeniorhynchus* were used in the genetic experimentation:

a. *Ravi bank;* Wild-type parent colony.

b. *White-eye* (w): Sex-linked recessive mutant with full penetrance and complete expressivity, showing 5.05± 0.17 per cent crossing over with sex locus.[5]

c. *Red-spotted eye* (Rs): A conditionally codominant mutant showing itself in white eye background. Located on the linkage group II.[4]

d. *Dieldrin-resistant* (D1): Newly isolated homozygous mutant stock.

e. *Dieldrin-white* (D1-w): Homozygous for the two mutants Dieldrin-resistance and white eye.

f. Dielddrin-Red spotted (D1-Rs): Homozygous for D1,w and Rs mutants.

The techniques used for various experimental crosses were similar to Baker[3]. Mass mating was allowed for all the crosses but individual rafts which represent single families were kept in separate vials. After hatching, percent hatched was recorded and single families were reared individually in flasks. The larvae were fed with liver powder every morning. Families showing less than 50% larval survival to adults were exlcuded from the final analysis of data where partial larval mortality might affect the quantitative assessment of gene segregation.

Both F_2 and backcrosses were designed to show both the mode of inheritance of dieldrin-resistance and its linkage relationship. Little atempt

was made to collect the F_1 data; however, egg rafts were constantly checked for the heterozygous genotype. The scores for D1 were obtained by treating fourth instar larvae with 0.125 PPM dieldrin, while other traits were assessed in the adults emerging from survivors of the dieldrin test. Thus half of the total segregation data were utilized in subsequent analysis.

RESULTS

The F_2 data obtained from the cross between dieldrin and wild-type is presented in Fig. 1, as cumulative larval mortality plotted against time. From this three larval populations can be distinguished. About 25% of the larvae dying in the first two hours, represented in the first sharp rise in mortality rates are the +/+ larvae. The second sharp increase after a short plateau, which includes about 50% of all larvae, represent the D1/+ group. The survivors of the 24-hour treatment are D1/D1, constituting about 25% of the total larval population. The actual scores of the three genotypes (+/+ 128,D1/+ 262 and D1/D1 144) conform with the 1:2:1 genetic ratio (where $\chi^2 = 1.15$, $P = 0.05 - 0.70$). This suggests that the D1 is inherited as a simple incompletely dominant gene showing a normal Mendelian segregation pattern with full penetrance and constant expressivity.

The results of the preliminary backcrosses of dieldrin-white heterozygous F_1 female with white-eye male are presented in Table I. These results indicate that D1 shows an independent assortment with white eye with recombination χ^2 1.20 ($P = 0.70 - 0.80$), 12.00 ($P < 0.01$), 1.87 ($P = 0.50 - 0.70$) and 2.89 ($P = 0.30 - 0.50$) for cross No. A, B, C, and D, respectively. The recombination χ^2 value is evidently high in cross B but previous observation of complete linkage in *C. tritaeniorhychus* female lends support to a possible hypothesis of independent assortment between D1 and w.[4] Whiteeye is a sex-linked gene located 5.05 ± 0.17 map units away from the sex locus[5]. D1 is not a sex-linked gene, and hence, must be located on some autosome.

The pooled segregation data between D1 and Rs is presented in Table II. The backcrosses with double heterozygous, dieldrinred spotted, F_1 female show a complete absence of D1-Rs recombinants (cross No. A & B). In cross C, however, a single D1-Rs recombinant appeared. This D1-Rs male might be spurious but even for a recombinant, the percent recombination is low. Since the Rs is a conditionally codominant mutant mapped on linkage group II[4] it is inferred that D1 locus might likewise be located on linkage group II.

The segregation data on the double heterozygous F_1 dieldrinred spotted male with D1 and Rs both in coupling and repulsive phase show the appearance of recombinant type D1-Rs individuals (cross No. D,E,F,G.). The

recombination χ^2 is high in these crosses suggesting the presence of linkage between two genes. The estimation of linkage between D1 and Rs by the method of maximum likelihood gave the recombination percentage value, ranging from 23.9% to 29.2%, in different crosses (i.e., cross D, 23.9; cross E, 29.2; cross F, 25.7; cross G, 27.1). The combined recombination value for the four crosses is calculated to be 26.46 ± 1.26%.

The percentage of larval hatching and development to adults was within normal limits in all the crosses.

DISCUSSION

The results of various experimental crosses indicate that dieldrin-resistance in *C. tritaeniorhynchus* is controlled by a single autosomal incompletely dominant mutation, which conforms with Mendelian segregation. The recombination data indicate that the dieldrin locus is located in the linkage group II 26.46 ± 1.26 map units away from Rs locus. However, more linkage data are needed for D1 to determine its linkage relationship with other known loci of linkage II i.e. ebony and alkaline phosphate-Fast.[6,7] However, experiments with radiation-induced cytological markers have suggested that Rs might be located on the extreme tip of the short arm (2L) of the sub-metacentric chromosome II.[6] Altogether these data suggest that D1 might be located between Rs and alkaline phosphate-Fast loci. The genetics of dieldrin-resistance has been studied in two other species of mosquito, *Aedes aegypti*[2] and *Anopheles quadrimaculatus*.[1] Dieldrin-resistance in both species is inherited as an autosomal incompletely dominant mutant. It is interesting to note that in all the species of mosquito in which genetics of dieldrin-resistance has been investigated, including that of *C. tritaeniorhynchus*, D1 has been assigned to linkage group II.

The presence of complete linkage in heterozygous C. tritaeniorhynchus female is also repeated between D1 and Rs in conformity with the findings of Baker and Rabbbanni[4], Baker and Sakai[8] and others. This is a unique biological phenomenon. Two other species of mosquito *Culex pipiens*[9,10] and *Aedes aegypti*[11,12] in which adequate linkage data are available, show recombination in both sexes, with some reported variations. The absence of crossing-over has been reported by Gowen and Gowen[13] in male *Drosophila, melanogaster*. Complete linkage is known to occur in male *Drosophila* and female silkworm. However, male *drosophila* and female silkworm are both heterogametic as compared to the homogametic female C. tritaeniorhynchus[14,3].

It appears that *C. tritaeniorhynchus* is sensitive to a much smaller dose of dieldrin. In *C. tritaeniorhynchus* 0.125 PPM dieldrin can differentiate between three genotypes, while in *Anopheles quadrimaculatus* 2.5 PPM is

required for such a separation.[1] Our observation suggests that in this species of mosquito, a minimal dose of 0.05 PPM can kill all sensitive and heterozygous resistant larvae. The application of minimal doses in nature will lessen pollution problems as well as damage to non-target species. Biologists realize that no one vector control method is completely effective *per se*. This presages the need for developing an integrated control involving physical, chemical, biological and genetic approaches. This is where a known minimum effective dose of dieldrin can be optionally useful.

SUMMARY

The methods for the isolation and selection of dieldrin-resistant gene are described. Preliminary data on the inheritance of dieldrin gene in *Culex tritaeniorhynchus* show that it is an incompletely dominant mutation, located on linkage group II, with a distance of 26.46 ± 1.26 map units from the red-spotted locus. The dose of dieldrin which can differentiate between three F_2 genotypes as well as the minimum lethal dose has been determined in this mosquito species.

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Table 1. Pooled segregation data between dieldrin-resistance (D1) and white-eye (W) in the double heterozygous F_1 *Culex tritaeniorhynchus* female. The parental genotypes are so written that the numerator represents the gene received from the female parent, while the denominator is that received from the male parent.

Cross No.	Proposed Parental Genotype	Adult Phenotype					χ^2	
		Dieldrin sensitive	Dieldrin Resistant				1:1:1:1	Oe
			White-eye		Normal eye			
	♀♀ ♂♂		♀	♂	♀	♂		
A	$\frac{W}{+}\frac{+}{D1} \times \frac{W}{W}\frac{+}{+}$	149	38	38	36	30	1.20	0.13
B	$\frac{+}{W}\frac{D1}{+} \times \frac{W}{W}\frac{+}{+}$	176	25	46	36	48	12.00	1.32
C	$\frac{W}{+}\frac{D1}{+} \times \frac{W}{W}\frac{+}{+}$	227	63	74	59	67	1.87	2.12
D	$\frac{+}{W}\frac{+}{D1} \times \frac{W}{W}\frac{+}{+}$	163	32	45	42	39	2.89	0.08

Table II. *Results of various crosses involving dieldrin-resistance (Dl) and Red-spotted eye (Rs) in Culex triataeniorhynchus.*

Cross No.	Proposed Parental Genotype ♀♀ ♂♂	Adult Phenotype: Dieldrin Sensitive	Dieldrin Resistant: Non-Spotted ♀♂	Dieldrin Resistant: Non-Spotted ♂♂	Dieldrin Resistant: Red-Spotted ♀♂	Dieldrin Resistant: Red-Spotted ♂♂	Percent Larval Rearing	Dieldrin Sex		Recombination
A	$\frac{Dl}{+}\frac{+}{Rs} \times \frac{+}{+}\frac{+}{+}$	806	367	338	–	–	91.4	0.03	1.19	–
B	$\frac{+}{Dl}\frac{Rs}{+} \times \frac{+}{+}\frac{+}{+}$	1,016	440	481	–	–	91.3	0.19	1.82	–
C	$\frac{+}{Dl}\frac{Rs}{+} \times \frac{Dl}{Dl}\frac{+}{+}$	372	123	136	–	–	55.7	14.18	0.75	–
D	$\frac{+}{+}\frac{+}{+} \times \frac{+}{Dl}\frac{Rs}{+}$	727	231	260	74	80	80.1	0.86	1.90	88.7
E	$\frac{+}{+}\frac{+}{+} \times \frac{Dl}{+}\frac{+}{Rs}$	602	205	219	79	96	78.9	0.01	1.64	51.3
F	$\frac{+}{+}\frac{+}{+} \times \frac{Dl}{+}\frac{Rs}{+}$	573	193	182	68	62	94.0	0.05	1.90	51.8
G	$\frac{+}{+}\frac{+}{+} \times \frac{+}{Dl}\frac{+}{Rs}$	698	237	216	87	81	91.8	0.02	1.17	65.9

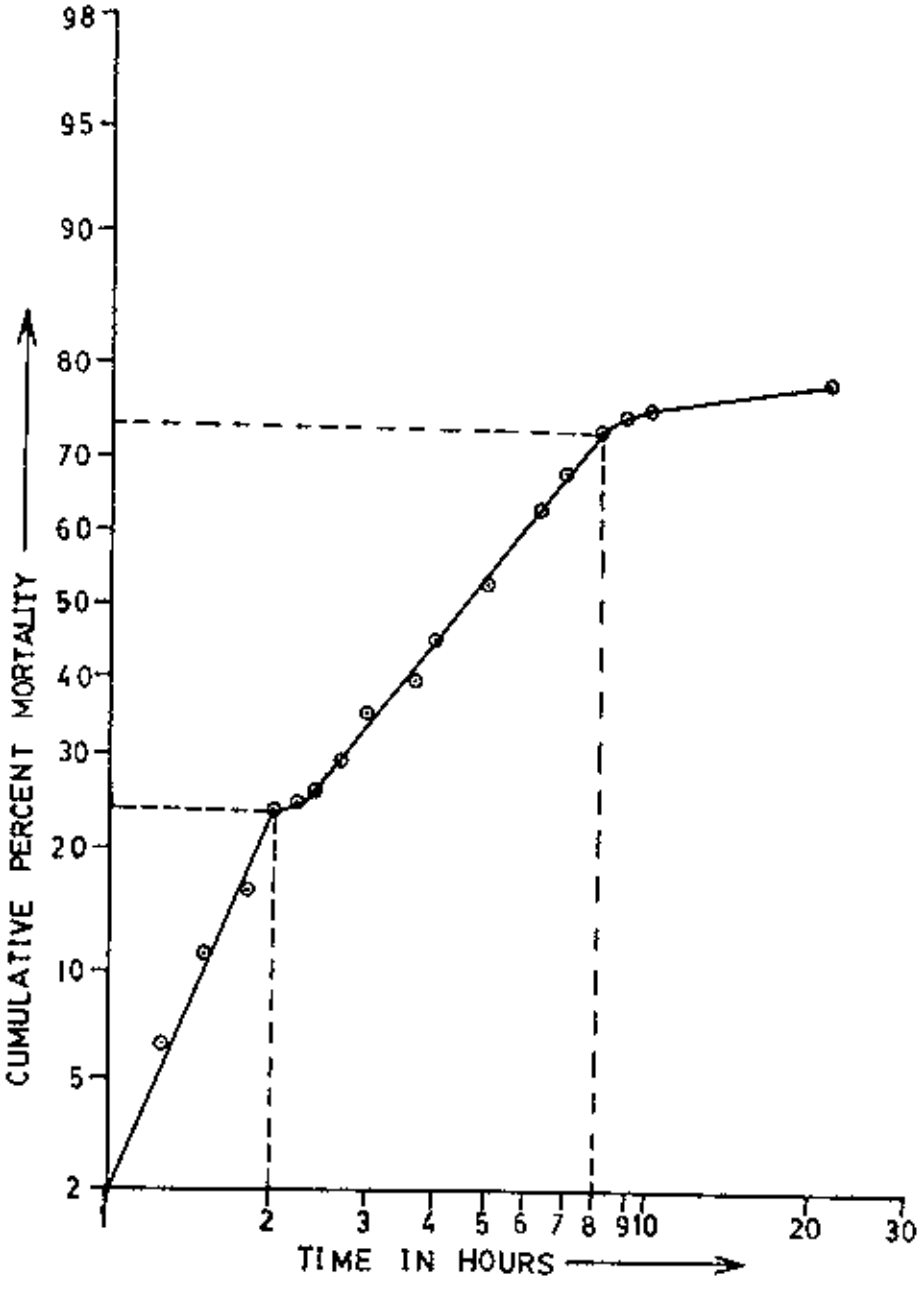

Fig. 1. Time cumulative mortality line of F_2 larval population in *Culex tritaeniorhynchus*, following its exposure to 0.125 PPM dieldrin plotted on log scale.

HISTOLOGICAL AND HISTOCHEMICAL STUDIES OF THE SPARGANUM OF *SPIROMETRA RANARUM* (MEGGITT, 1925) IN *RANA CANCRIVORA* GRAVENHORST, 1829*

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ABSTRACT

The histological structure and organization of the sparganum of *spirometra ranarum* are presented. The cuticle consists of three elements: the cuticular hair, homogeneous layer and basement membrane. Immediately beneath is the subtegumentary musculature (peristaltic layer) arranged into an outer circular and inner longitudinal layers; transverse muscle fibers are distributed through the loosely packed parenchyma. The spongy parenchyma, with large cells and compact nuclei, fill up all spaces not occupied by the fibro-muscular bundles, thick-walled excretory ducts, minute flame cells and nervous tissues. Gland-like vacuolated cells are distributed in the entire length of the sparganum; laminated calcareous corpuscles are found throughout the sparganum except one cm from the anterior and at the invaginated region. The nerve bundles are noted near excretory ducts.

Histochemical tests showed: (1) greatest concentration of glycogen in the muscle layers and parenchyma cells, less in the other subtegumentary cells, and very slightly positive in the peripheral layer of the calcareous corpuscles in all regions; (2) the tegument and the calcareous corpuscles reacted very strongly to Fischler's method for fatty acids, and less in the muscle layers in the same structures at the anterior portion and the posterior regions; (3) with the alkaline phosphatase test[1] (Gomori, 1952, 1956) the calcareous corpuscles reacted very strongly, but slightly positive in the cuticle and the middle portion. The muscle layers at the middle and posterior regions had more acid phosphatases, less in the tegument at the middle region, and almost nil at the anterior region; (4) more concentration of acid mucopolysacharides in the parenchyma and less in the circular and longitudinal muscle layers at the anterior region; (5) the medullary parenchyma of the proliferative and growing areas of the anterior region reacted very strongly to the RNA (Methyl-green-pyronin) test, less in the same structure at the middle region and longitudinal muscle layer in all regions; the slight reaction of the circular muscle layer in all regions including the medullary parenchyma at the posterior region indicate less proliferaive activities.

*Submitted in partial fulfillment for the degree of M.S. Zoology, University of the Philippines under the direction of Dr. Carmen C. Velasquez, Professor of Zoology, University of the Philippines, Diliman, Quezon City.

INTRODUCTION

Bonne[2] in the Netherlands East Indies (Malayan Archipelago, Malaya, Java and De Hartogh) experimentally infected mice, monkeys and frogs by feeding them with procercoid infected with *Cyclops* sp. and spargana from frogs and tadpoles. Furthermore, spargana, he obtained from man and monkey developed onto *Spirometra ranarum* when fed to cats.

Spirometra ranarum (Meggitt, 1925) has received very little attention in this country.

In the Philippines, Africa[3] experimentally infected *Cyclops* (*Eucyclops*) *serrulatus* Fisher and *Cyclops* (*Micro-cyclops*) *bicolor* G.O. Sars with coracidia of *Diphyllobothrium mansoni* (Cobbold, 1882). Tubangui[4] described *Diphyllobothrium mansoni* obtained from dogs and cats. Tubangui's[5] checklist include *Rana vittigera* Wiegman, 1835 and probably other species of frogs as second intermediate hosts, of the above named species. The locality records indicate Manila, Los Baños, Laguna, Luzon Island, and in the Visayan Islands in Palo, Leyte. Yutuc[6] described the life cycle of *Diphyllobothrium erinacei* (Rudolphi, 1815) using eggs obtained from feces of infected dogs and cats from neighboring areas in Quezon City, Luzon Island, Philippines. The incidence of sparganosis as reported by Yutuc[6] in the frogs obtained from Pandacan, Manila, Pasig, Rizal, Laguna, Lubao and San Fernando in Pampanga and Labrador, Pangasinan was 12.1%. Laguna showed the highest degree of infection, being 15 positive out of 24 frogs he examined.

Unfortunately, however, there is no available record of the incidence of the sparganum of *Spirometra ranarum* in frogs in this country, this being the first report on the species in the Philippines.

Despite the medical and veterinary importance of sparganosis, no further investigation has been done in this country much less on the physiology of any pseudophyllidean sparganum.

Vik[7] recognized the necessity of exploring the different areas in pseudophyllidean cestode host-parasite relationship. Recent investigations suggest that, although the spargana of the oriental form, *Spirometra ranarum* is generally regarded as morphologically similar to that of the American form *Spirometra mansonoides*, closer study reveal important physiological and behavioral differences[8].

Spargana from *Rana cancrivora* from Kuala Lumpur, Malaysia sent to Mueller in 1964 by Dr. Klaus Rehde[8] were identified as *spirometra ranarum* after propagation for a year. He observed that the sparganum of the oriental form . . .

> ". . . moves in a characteristic way; the scolex sets up peristaltic waves in rapid succession, so that the anterior end often appears saggitate or

moliniform; *Spirometra mansonoides*, on the other hand, produces longer, slower waves, and the first tends to fade out before the second begins."

"... tight encapsulation into yellowish cysts at the end of six or eight months,"

Furthermore, he attributed to the steady increase in growth rate measured by the length of the sparganum of *Spirometra ranarum* for four months and to the greater antigenic property of *Spirometra ranarum* arousing a greater response on the part of the mouse. In contrast, *Spirometra mansonoides* continued to increase in length though variable for ninety-nine weeks. Very recently Mueller[9] tried to compare the growth promoting effect of *Spirometra mansonoides* vs: 3 oriental forms one from Australia, Malaya and Taiwan. They seem to have

"... elusive qualitative differences," closely resembling each other but differ from *Spirometra mansonoides* in the character of the uterus. Preliminary investigation yielded inconsistencies between his 1970 results and the original experiments in 1966, using the Malay form. Unfortunately, lack of materials impeded the progress of his work. The above physiological and behavioral differences may be further elucidated by using certain histochemical techniques.

Though certainly a different species from *Spirometra mansonoides* as Mueller[8] has said:

"... its exact nomenclature status," is uncertain. Vik and Anderson[10] suggest the importance of the larval form in the classification of some pseudophyllideans.

From the available literature (von Brand, 1966), it is evident that very few histochemical studies on glycogen distribution has been done on cestodes and less on pseudophyllidean cestodes, much less on the plerocercoid. No tests have been done either lipid or fatty acids contents of *Spirometra ranarum*.

Fairbairn (1960) (von Brand, 1966) pointed out that destruction due to penetration or migration may lend to the production of substances potentially useful to parasite nutrition. He maintained that penetration necessarily involves enzymatic activities from parasites. A tissue penetrating enzyme of protozoa and parasitic helminths has been called hyaluronidase. No data is available for any pseudophyllidean plerocercoid, a tissue penetrating form.

Proteins are concerned with the process of muscle contraction, supporting and protective function, enzyme action, production of antibodies and toxins. Protein content and fractionations have been calculated in many cestodes but none is available for *Spirometra*. The distribution of such proteolytic enzymes in the sparganum, a tissue penetrating phase in the life cycle of *Spirometra ranarum* needs study.

Very recently, numerous publications on histochemical studies have been done, but mostly on adults and comparatively very little on the larvae. No studies on the antero-posterior gradient of RNA synthesis are available for *S. ranarum*.

This investigation aims to demonstrate, in the sparganum of *Spirometra ranarum* the presence and distribution of glycogen, fatty, acids, ribonucleic acids, acid mucopolysaccharides, acid and alkaline phosphatases by certain histochemical techniques.

MATERIALS AND METHODS

At intervals from August, 1969, for a period of one year, *Rana cancrivora* from rice paddies in Los Baños, Laguna and Malabon, Rizal were collected and dissected for plerocercoids.

Spargana or plerocercoids were usually found in the muscles of the leg and rarely in the abdominal and pectoral muscles. The spargana were removed from the muscles by fine forceps and placed directly in the various fixatives. The spargana recovered from *Rana cancrivora* that of *Spirometra ranarum* is behaviorally and morphologically different. The scolex of the sparganum of *Spirometra ranarum* (Fig. 1) is spatulate with wrinkled anterior end and very active, setting up peristaltic waves in rapid succession as those described by Mueller[8]. To get the adults for specfic identity, spargana were fed to cats and found to be that od *Spirometra ranarum*. The spatulate scolex or "holdfast" organ is 0.68 mm long, and 0.85 mm maximum width in my mounted specimen. (Fig. 2). The relaxed strobila is 49 mm long. Some gravid proglottids are longer than brond (Fig. 3), others are square (Fig. 4) depending upon fixation procedures. There are three or four outer uterine coils, the posterior, broader and more dilated than the terminal. The rosette inner coils are distinct; ovary dendritic; testis and vitellaria in bands on lateral margins of the worm, fused or unfused at the antior median line. Male genital aperture is anterior to the female vaginal aperture.

Histological sections were prepared from spargana fixed in Bouin's fluid. The paraffin method was used and sections were cut at 7 microns, subsequently stained in Delafield's hematoxylin, counterstained with eosin and Mallory's triple stain.

Tests for glycogen, fatty acids, acid and alkaline phosphatases, acid mucopolysaccharides and ribonucleic acid were done.

Test for Glycogen

Spargana were fixed in Gendre's fluid at -73°C (dry carbon dioxide) prior to the Periodic Acid Schiff (PAS) and Best's carmine tests. Spargana of about 5 to 9 cm long were cut from anterior to the posterior end at intervals of one cm. Experimental and control sections were 8 microns thick. Saliva was used for the diastase-control sections maintained between 30° to 34°C for one hour before the tests.

Test for Fatty Acids

Modified Fischler's method was used with 10% formalin-fixed spargana. Frozen sections were cut at 20 microns. Control sections were previously extracted for 24 hours with 3 changes of alcohol/ether (equal parts) before staining.

Test for Acid and Alkaline Phosphatases

The lead nitrate method for acid phosphatase (after Gomori, 1950) was used. The spargana were fixed in cold acetone (about 4°C). NaF at 0.01M concentration was used for the control sections.

For alkaline phosphatases, the modified Gomori method (after Fredericsson, 1952, 1956) was used. The spargana were fixed for 12 hours in 90% ethyl alcohol at about 22°C and post fixed in 90% ethyl alcohol at the same temperature for another 12 hours. Control sections were treated with KCN at 0.009M concentration, pH 8-9.

Test for Acid Mucupolysaccharides

Lillie's (1954) (Pearse, 1960) alcohol lead nitrate fixative was used. Spargana were kept in the fixative for 24 hours at room temperature between 26.6° to 33°C. The modified Alcian blue method for acid mucopolysaccharides (after Steedman, 1950) was used. Control sections were treated overnight with $FeCl_3$ at 0.05% before the experiment procedures were done.

Test for Ribonucleic Acid

The methyl-green pyronin method for RNA (Brachet, 1942) (Pearse, 1960) was used for AAF (after Lillie, 1949) (Pearse, 1960) fixed spargana.

The control sections were immersed in 0.15 M NaCl solution for 5 hours at 37°C. before the test.

In all tests 2 trials were done.

RESULTS AND OBSERVATIONS

Histology

The histological structure and organization of the sparganum of *Spirometra ranarum* resembles the general pattern of *Ligula* sp. as interpreted by Blochmann (1835) and *Spirotrema mansonoides*[11] Mueller, 1935.

In my sections stained in Delafield's hematoxylin and counterstained with eosin and in Mallory's triple stain, the tegument (Figs. 12 and 14) vary in thickness from 5 to 7 microns at different regions antero-posteriorly. The tegument stained blue in Delafield's hematoxylin and lighter blue in Mallory's triple. Doubling of the tegumentary layer (Fig. 14) is found in and immediately posterior to the invaginated region which in whole spargana appear as an elongated funnel-like structure. The three elements of the tegument are the (1) "cuticular hairs" (Figs. 11 & 13), 2 to 3 microns long depending upon the fixative and staining procedures; (2) the homogeneous layer of about 2 microns thick (Fig. 11), and (3) basement membrane of about 2 to 3 microns (thick (Fig. 11). Just below the basement membrane is the subtegumentary (peristaltic layer) 5 to 6 microns thick consisting of an outer tegumentary and inner longitudinal layers (Figs. 11, 12 & 13); both muscle layers are well developed axially from anterior to posterior end. The transverse muscle fibers (Fig. 8) are scattered randomly through the loosely packed parenchyma. Scattered in the parenchyma are large cells, 15 to 23.3 microns, with prominent, compact nuclei, 2.5 to 5 microns. They are slightly round or ellipsoid (Fig. 6), and stained blue in both Delafield's hematoxylin and Mallory's triple stain. In addition, fibre-muscular bundles are well distributed and at times arranged perpendicular to one another along the axis of the worm (Fig. 9). Also seen, are excretory ducts of varying sizes with thickened walls, 3.3 to 6.7 microm. (Fig. 9). These ducts and minute flame cells 3.3 to 5 microns long, (Fig. 9), are believed to have osmoregulatory functions. The vessels were observed in alive specimens of *Spirometra mansonoides* by Mueller[8]. In sections stained in both Delafield's hematoxylin and Mallory's triple stain, numerous large, round or elliptical gland-like vacuolated cells 16.7 to 25 microns, are found in the entire length of the sparganum (Figs. 8 & 11).

Fig.10 shows the concentric laminated layers of the calcareous corpuscles, 16.3 to 25 microns in diameter and stained blue with Delafield's

hematoxylin and Mallory's triple. Numerous calcareous corpuscles are distributed throughout the sparganum except at one cm from the anterior end at the invaginated region.

The nervous system is seen as bundles of nerve fibers near excretory ducts (Fig. 9). These structures are comparable to those described by Mueller[11] for *Spirometra mansonoides*. However, since the structure of the nervous system of plerocercoids are relatively unknown, a special technique and study is recommended.

HISTOCHEMICAL STUDIES

Distribution of Glycogen

The results of the Best's carmine method and Periodic Acid Schiff (PAS) tests for glycogen are in Table I. All the microphotographs are from Acid Schiff (Figs. 15, 16 and 17) tests.

In the (PAS) test, the presence of glycogen with varying color intensities from red to pink was used as criteria for the presence of glycogen.

Very strongly positive for glycogen are the circular, longitudinal and transverse muscle layers and the parechyma cells not only in the scolex but through the entire length of the sparganum. Strongly positive but of lesser intensity are the tegument and other subtegumentary cells. Slightly positive for glycogen is the concentric peripheral layer of the calcareous corpuscles. The color was not completely removed from the medullary parenchyma of the control sections.

Distribution of Fatty Acids

The fatty acids were determined by Fischler's method (Pearse, 1960) any structure staining black or blue was interpreted as fatty acids. The results are indicated in Table I.

The tegument at the anterior region (Fig. 18) stained blue (positive), dark blue (strongly positive) to almost black (very strongly positive) at the middle and posterior regions of the sparganum (Figs. 19 & 20) respectively. The color in all cases is diffused, giving the same reactions for the circular, longitudinal and transverse muscle fibers. It is slightly positive in the parenchyma immediately adjacent to the subcuticular part at the middle and posterior regions. The network of vessels stained blue (Figs. 19 & 20).

Distribution of Acid and Alkaline Phosphatases

The results are shown in Table II. The presence or absence of acid phosphatases are based on the various deposit of lead sulphide and cobalt sulphide for alkaline phosphatases as indicated by varying staining intensities of black.

The tegument of the middle portion and the muscle layers of the middle and posterior regions of the sparganum are strongly positive for acid phosphatase at pH 5 to 6. But the tegumentary elements of the anterior region reacted slightly (Figs. 21, 22 and 23).

Very strongly positive for alkaline phosphatases are the calcareous corpuscles at the middle and posterior regions and slightly positive at the cuticular and subcuticular elements excluding the muscular layers (Figs. 24, 25 and 26).

Distribution of Acid Mucopolysaccharides

The results are shown in Table II. The parenchyma of the anterior region reacted very strongly to the Alcian blue test, but the longitudinal and transverse muscle layers gave slightly positive reaction (Figs. 27 & 28).

Distribution of Ribonucleic Acid (RNA)

The varying intensities from red to pink in the methyl-green-pyronin method (Brachet, 1942) (Pearse, 1960) were used as criteria for the presence of RNA. The results are shown in Table III.

The parenchyma cells of the scolex reacted very strongly to the test (Fig. 29). Lesser staining intensity was noted in the parenchyma cells at the middle region of the sparganum and longitudinal muscle layer of the entire worm (Figs. 30 & 31). The circular muscle layer of the entire worm, and parenchymal cells at the posterior portion were slightly positive (Figs. 29, 30, 31, 32 & 33).

DISCUSSION

Histology

Because of the lack of a mouth and an alimentary canal, the body surface of the cestodes have aroused the interest of many workers. The function of the epidermis has taken a new significance and therefore the

elucidation of its structure and composition must of necessity lead us to the proper understanding of cestode physiology both in the larva and adult.

This investigation elucidates on the structure and composition of the sparganum of *Spirometra ranarum* within the limitations of available facilities.

Under the light microscope, the microvilli have not been observed in the sprganum of *Spirometra ranarum.* Studies on its ultrastructure if and when the facilities are available may eventually confirm the findings of others.

The thin tegumentary layer of the sparganum offer greater possibility for increased absorption capacity in the uptake of nutritive materials from the surroundings.

The well developed circular and longitudinal muscle layers from the anterior to the posterior may enhance the penetrating capacity of the worm through the intestinal wall of unsuitable definitive hosts to their muscles. These results in their migration to the different regions of the body of unsuitable definitive hosts.

Mueller's[11] illustrations showed the presence of excretory ducts in histological sections of normal specimen of the sparganum of *Spirometra mansonoides.* He indicated that the dorsal vessel can be differentiated from the longtitudinal by the thickness of the surrounding tissues: the network of vessels were well defined as seen in his illustration of a living sparganum of *Spirometra mansonoides*[8] He suggested that:

> ". . . perhaps the network of vessels in the scolex and elsewhere might serve the purpose of distributing the food materials, as well as an excretory function."

This view may probably hold true for the sparganum of *Spirometra ranarum* where the concentration of these thick-walled ducts were more in the medullary than the cortical region.

In an attempt to elucidate on the functions of the different regions of the sparganum of *Spirometra ranarum,* histochemical tests have been done.

Glycogen

The heavy glycogen deposits as determined by the PAS and Best's carmine methods in the muscular layers, and parenchyma of *Spirometra*

ranarum plerocercoid confirm the finding[12] of Takahashi (1959) on the plerocercoid of *Diphyllobethrium mansoni*. There seems to be no difference in the amount of glycogen at the anterior, middle and posterior regions of the worm, glycogen gradient does not occur along the larval strobila.

The PAS and Best's carmine methods gave a doubtful positive reaction for the tegument. In paraffin sections,[1] Leblond et al. (1957) consider that once glycogen has been removed by diastase, only one class of materials gives a positive PAS reaction, and are referred to as carbohydrate-protein complexes.

Apparently, in the plerocercoid, this reserve carbohydrate is utilized to maintain the normal bodily functions as in other animals. In *Taenia taeniseformis* and its larva *Cysticerous fasciolaris* Rudolphi (1808) no quantitative gradient along the strobila of *Taenia taeniaformis* was observed, but that the larger individual proglottid had more calcareous corpuscles than the small ones[13]. He concluded that more corpuscles disappear during anaerobic than aerobic conditions and subsequently believes that these corpuscles serve to buffer acids entering the body of the worm from the outside. In the plerocercoid of *Spirometra ranarum* being a tissue phase in the life cycle, the corpuscles may serve to buffer acids entering the body as was found by von Brand (1966) in *Taenia taeniaformis*. The presence of calcium, magnesium, phosphorus and carbon dioxide in *Spirometra mansonoides* larva by qualitative isolation on tissues with alkali and subsequent centrifugation has added to our knowledge of calcareous corpuscles in the pseudophylidean cestodes[14]. They further showed a smaler variety in the minor components of the larva than those isolated from adult worms. They believed that;

> ". . . the differences is related to the greater uniformity in the surroundings of the larval worms as compared to the intestinal lumen."

This selective reaction indicates that the animal draws energy from reserve carbohydrates in the form of glycogen; some convert this reserve into glucose to maintain normal body functions. The saliva-digestible polysaccharides in my sections as demonstrated by PAS reaction is predominantly along the concentric peripheral layer of the calcareous corpuscles. Histochemical tests offer a way of investigation for small organisms, but give only a semi-quantitative result.

Fatty Acids

Von Brand's (1966) hypothesis, that the presence of fatty acids in the osmoregulatory vessels in the sparganum of *Spirometra ranarum* may well be waste products from carbohydrate fermentation, although questioned, may

elucidate this phenomenon. On the other hand, the positive reaction in the tegument of my specimens although not supported by experimental evidence, may indicate that fatty acids are obtained by absorption from the surroundings.

Further studies may elucidate on the presence of fatty acids in the tegument of the sparganum of *Spirometra ranarum*.

Acid and Alkaline Phosphatases

In the plerocercoid of *Spirometra ranarum*, the cuticular and subcuticular layers at the anterior region reacted very weakly to alkaline phosphatases test as compared to that of the middle and posterior regions. The localization of enzymes in the nerve cord is not clear with the modified Gomori method (after Fredericsson, 1952, 1956) (Pearse, 1960). From the results, the active phosphorylation is at the middle and posterior regions of the worm. This probably is correlated with the active movement at this region during migration and when disturbed mechanically.

The lead nitrate method for acid phosphatases[1] (after Gomori, 1950) was found to have variable reactions even in the same structure at the same region or in different parts of that region. However, the test shows a very slightly positive reaction in the tegumentary and subtegumentary layers of the anterior region and around the scolex, but more in the middle and posterior regions. This variable distribution of the black precipitate is explained by Pearse[1] in that the Gomori method gives only an approximate or topographical picture of the distribution of acid phosphatases in the tissues.

Acid Mucopolysaccharides

The strongly positive region of the parenchyma of the scolex exclusive of the nuclei suggests the origin of the acid mucopolysaccharides and their active function in the penetration of the parasite through the intestinal wall of unsuitable definite hosts. Hyaluronidase proper is a mucopolysaccharidase which does not occur in typical form in skin penetrators as in the cercariae of *Schistosoma mansoni* and larvae of *Strongyloides ratti*[15].

There is no direct histochemical method available at present for the hyaluronidase. The Alcian blue method for the acid mucopolysaccharides imply the localization of a protease in the structures of the scolex positive for such a test. However, the active enzyme may explain its selective capacity to penetrate the intestinal wall since the larva localize under the skin, remains there until eaten by a suitable host.

Ribonucleic Acid

The Brachet (1942) method[4] for the localization of ribonucleic acid (RNA) synthesis in tissues, reflects the high level of this activity in those areas.

The very strong positive reaction of the medullary parenchyma in the anterior region especially in the scolex shows this region as proliferative and actively growing areas. Decreasing reaction in the longitudinal muscle layer in the anterior and middle regions and almost negligible in the circular muscle layer in all regions indicate that there is an axial gradient and localization of RNA with respect to growth and development in the sparganum of *Spirometra ranarum*.

It is evident that medullary parenchyma grows more rapidly at the region of the scolex and contributes to the organization and development of the sparganum. The middle and posterior portions probably aid in the nutrition of the anterior region as shown in the tests.

Biochemical studies may provide quantitative analysis of the growth rate in different regions.

SUMMARY

1. The detailed histological organization of the sparganum of *Spirometra ranarum* is presented.

2. Similar observations have been made on the histology of *Spirometra ranarum* as those of *Spirometra mansonoides* by Mueller[11].

3. The histological structures and organization at the region of the scolex is described; the prominent tegumentary folds and rather deep groove are results of invagination in this region.

4. The three elements of the tegument namely, cuticular hairs, homogeneous layer and basement membrane were observed in the entire length of the sparganum.

5. Beneath the tegument is the subtegumentary or peristaltic layer consisting of an outer circular and an inner longitudinal layers. Transverse muscle fibers are distributed in the parenchyma, with large vacuolated cells containing compact nuclei. The fibro-muscular bundles, arranged perpindicular to one another, the thick-walled excretory ducts and minute flame cells, and gland-like vacuolated cells were observed in spaces not occupied by the parenchyma in the entire length of the sparganum. The laminated

calcareous corpuscle are distributed throughout the sparganum except one cm from the anterior end at the invaginated area. The nerve bundles are usually seen near excretory ducts.

6. The presence of large amounts of glycogen in the muscle layers and parenchyma cells of the entire worm were determined by the Best's carmine and PAS tests, and less in the other subtegumentary cells.

7. The tegument in the middle and posterior portions reacted very strongly to the fatty acids test; strongly positive are the muscle layers in the said regions; lightly positive are the cuticle and muscular layers in the anterior region and parenchyma cells in the middle and posterior regions.

8. Sites of enzyme activities namely: (1) the calcareous corpuscles, very strongly positive to alkaline phosphatases test and slightly positive in the tegument at the middle portion; (2) the muscle layers at the middle and posterior regions, and the tegument at the middle region reacted very strongly to the acid phosphatase test (after Gomori, 1950) and slightly in the tegument at the anterior region.

9. Acid mucopolysaccharides are found more in the parenchyma and slightly in the circular and longitudinal muscle layers at the anterior region.

10. The RNA test, although spotty and qualitative indicates an axial gradient of growth in active and proliferative areas antero-posteriorly.

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Table I. Results of the Histochemical Tests on the Sparganum of *Spirometra ranarum from* the Muscles of *Rana Concrivora*

	Test for glycogen						*Test for fatty acids*		
	Periodic Acid Schiff (PAS)			*Best's Carmine*			*Fischler's method*		
	ant	*mid*	*post*	*ant*	*mid*	*post*	*ant*	*mid*	*post*
Tegument	+?	+?	+?	+?	+?	+?	–	–	–
cuticular hairs	–	–	–	–	–	–	+	+++	+++
homogeneous layer	–	–	–	–	–	–	+	+++	+++
basement membrane	–	–	–	–	–	–	+	+++	+++
Calcareous corpuscles	–	+*	+*	–	+*	+*	–	+++	+++
Subtegumentary layer									
Muscle layer	–	–	–	–	–	–	–	–	–
circular	+++	+++	+++	+++	+++	+++	+	++	++
longitudinal	+++	+++	+++	+++	+++	++	+	++	++
transverse	+++	+++	+++	+++	+++	+++	+	++	++
Parenchyma cells	+++	+++	+++	+++	+++	+++	–	+	+
other parenchyma cells	++	++	++	++	++	++	–	–	–

Legend:

+ –positive
++ –strongly positive
+++ –very strongly positive
– –negative
* –peripheral layer
–? –doubtful

Table II. Distribution of Some Enzymes and Acid Mucopolysaccharides in the Spargaum of *Spirometra Ranarum* from the Muscles of *Rana Cancrivora*

	Acid Phosphatases (After Gomori, 1950)			*Alkaline phosphatases (modified Gomori after Frederiksson 1952, 1956)*			*Acid mucopolysaccharides (after Steedman, 1950)*		
	ant	*mid*	*post*	*ant*	*mid*	*post*	*ant*	*mid*	*post*
Tegument	–	–	–	–	–	–	–	–	–
cuticular hairs	+	++	–	–	+	–	–	–	–
homogeneous layer	+	++	–	–	+	–	–	–	–
basement membrane	+	++	–	–	+	–	–	–	–
Calcareous corpuscles	–	–	–	–	+++	+++	–	–	–
Subtegumentary layer									
Muscle layer	–	–	–	–	–	–	–	–	–
circular	–	++	++	–	–	–	+	–	–
longitudinal	–	++	++	–	–	–	+	–	–
transverse	–	++	++	–	–	–	–	–	–
Parenchyma cells	–	–	–	–	–	–	–	+++	–
other parenchyma cells	–	–	–	–	–	–	–	–	–

Legend:

\+ –positive
++ –strongly positive
+++ –very strongly positive
– –negative

Table III. Distribution of Ribonucleic Acid (RNA) in the Sparganum of *Spirometra Ranarum* from the Muscles of *Rana Cancrivora*

	RNA		
	Methyl-green-pyronin method		
	anterior	*middle*	*posterior*
Tegument	–	–	–
cuticular hairs	–	–	–
homogeneous layer	–	–	–
basement membrane	–	–	–
Calcareous corpuscle	–	–	–
Subtegumentary layer			
Muscle layer	–	–	–
circular	+	+	+
longitudinal	++	++	++
transverse	–	–	–
Parenchyma cells*	+++	++	+
other parenchyma cells	–	–	–

Legend:

+ – positive
++ – strongly positive
+++ – very strongly positive
– – negative
* – medullary parenchyma

ABBREVIATIONS USED

b	basement membrane
cc	calcareous corpuscles
cp	cortical parenchyma
e	excretory duct
f	tegumentary fold
fb	fibro muscular bundle
fm	flame cell
g	gland-like cell
gp	genital pore
gp_1	male genital pore
gp_2	female genital pore
h	tegumentary hairs
hg	homogeneous layer
m_1	tegumentary muscle layer
m_2	longitudinal muscle layer
m_3	transverse muscle layer
mp	medullary parenchyma
n	nucleus
o	ovary
p	parenchyma cell
t	tegument
tc	terminal chamber
te	testis
u	uterus
v	vitellaria

EXPLANATION OF PLATES

PLATE I*

Fig. 1 Photograph showing the scolex of adult *Spirometra ranarum*.

Fig. 2 Part of the strobila of *Spirometra ranarum*.

Fig. 3 Gravid proglottid of *Spirometra ranarum*.

Fig. 4 Sparganum of plerocercoid found in the muscle of the leg of *Rana cancrivora*.

Fig. 5 The scolex of sparganum of *Spirometra ranarum*.

Fig. 6 Section through the anterior region of the sparganum showing the parenchyma cells with prominent nuclei.

Fig. 7 Section through the middle region of the sparganum showing the calcareous corpuscles.

Fig. 8 Section through the middle portion of the sparganum showing transverse muscle layer and gland-like vacuolated cells.

Fig. 9 Section through the middle portion of the sparganum showing excretory ducts, fibro-mascular bundles, flame cells and bundle of nerve cells.

Fig. 10 Enlarged portion showing excretory duct at the middle region.

Fig. 11 Section through the middle portion of the sparganum showing the cuticular hairs, circular, longitudinal and transverse muscle layers and gland-like vacuolated cells.

Fig. 12 Section through the middle portion of the sparganum showing the tegument and circular muscle layer.

Fig. 13 Section through the posterior portion of the sparganum showing the cuticular hairs, and gland-like vacuolated cells.

*The microphotographs are Delafield's hematoxylin stained.

Fig. 14 Section through the scolex of the sparganum showing the groove as the result of the invagination.

PLATE II

Fig. 15 Section through the scolex of the sparganum showing glycogen-positive areas.

Fig. 16 Section through the middle of the sparganum showing glycogen-positive areas.

Fig. 17 Section through the posterior part of the sparganum showing glycogen-positive areas.

Fig. 18[a] Section showing fatty acids-positive areas of the posterior portion of the sparganum.

Fig. 19[a] Section showing fatty-acids-positive areas of the middle portion of the sparganum.

Fig. 20[a] Section showing fatty-acids-positive areas of the posterior portion of the sparganum.

Fig. 21[b] Section through the scolex of the sparganum showing sites of acid phosphatases activity.

Fig. 22[b] Section through the middle portion of the of the sparganum showing sites of acid phosphatases activity.

Fig. 23[b] Section through the posterior region of the sparganum showing sites of acid phosphatases activity.

Fig. 24[c] Section through the scolex of the sparganum showing distribution of alkaline phosphatase-positive materials.

Fig. 25[c] Section through the middle of the sparganum showing distribution of alkaline phosphatase-positive materials.

Fig. 26[c] Section through the posterior region of the sparganum showing distribution of alkaline phosphatase-positive materials.

Fig. 27[d] Section through the scolex of the sparganum showing acid mucopolysaccharides-positive areas in parenchyma cells.

Fig. 28[d] Enlarged portion of Fig. 26.

Fig. 29[e] Section showing proliferative areas of the sparganum.

Fig. 30[e] Section showing RNA-positive areas in the middle portion of the sparganum.

Fig. 31[e] Section showing RNA-positive areas in the posterior portion of the sparganum.

Fig. 32[e] Enlarged portion of the subcuticular layer of the sparganum.

Fig. 33[e] Enlarged portion of the circular muscle layer of the sparganum.

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[a]Positive reactions to Fishler's method test for fatty acids are indicated.
[b]Positive reactions to Gomori's test for acid phosphatases are indicated.
[c]Positive reactions to modified Gomori's test for alkaline phosphatases are indicated.
[d]Positive reactions to acid mucopolysaccharides test for hyaluronidase are indicated.
[e]Postive reactions to methyl-green-pyronin test for RNA are indicated.

0.2mm
0.1 cm
0.5 mm
f
gp1
gp2
v
s
u
o
3
4
5
0.1 mm
cp
0.01 mm
g
m3
7
8
n
0.05 mm
0.04 mm
2
6
mp
9
b
ng
m1
m
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g
e
10
mp
p
cp
m1
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0.01 mm
cp
12
14

PLATE I

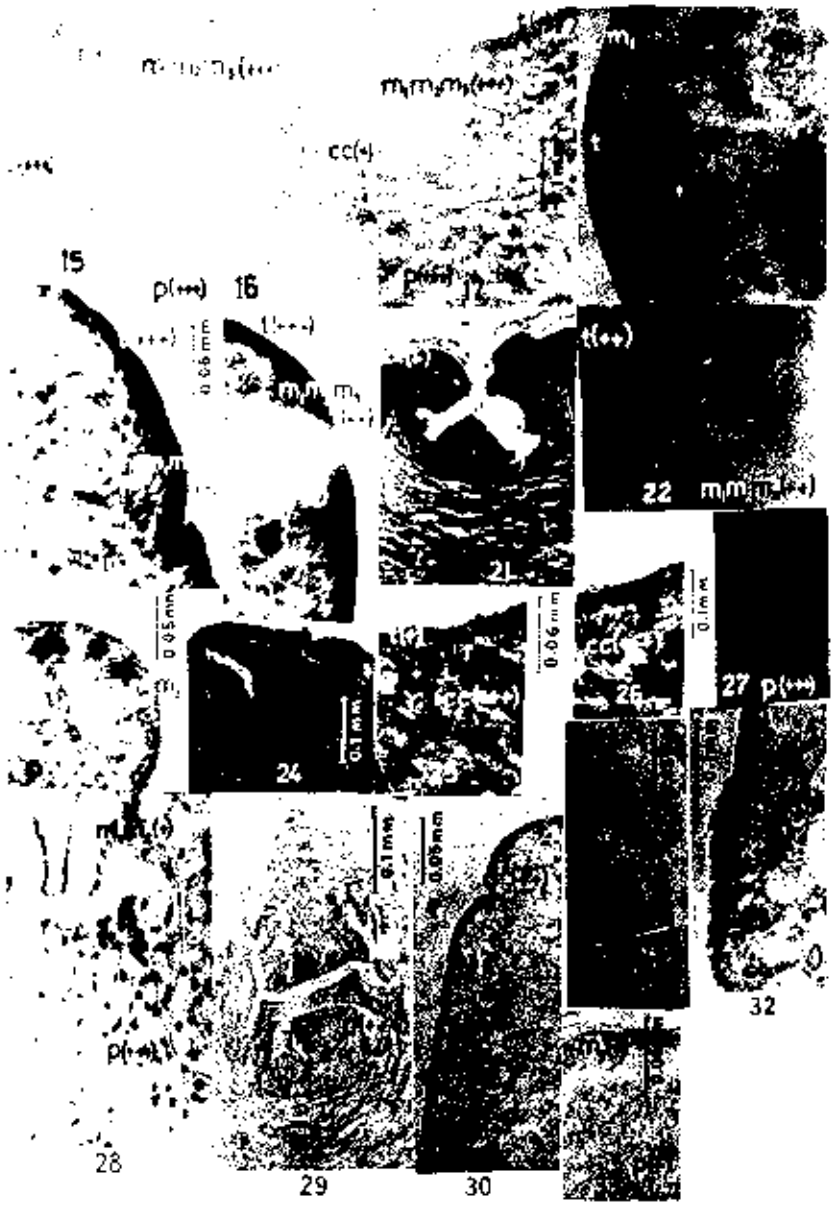

PLATE 2

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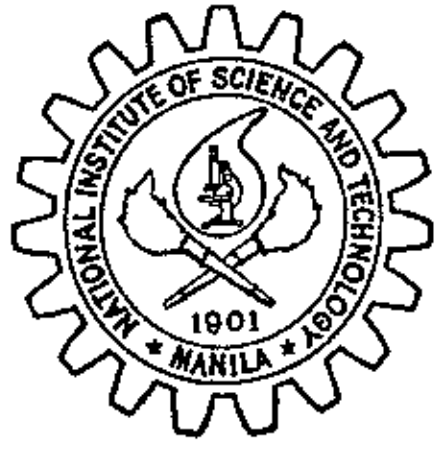

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